LABORATORY DIAGNOSIS
OF SYPHILIS

LABORATORY
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A *Manual for*
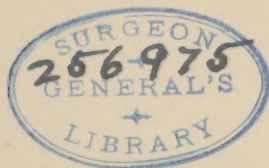### STUDENTS AND PHYSICIANS

BY

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FIFTY-NINE ILLUSTRATIONS OF WHICH
THIRTEEN ARE IN COLOR

❧

PREFACE

The laboratory procedures for the diagnosis of syphilis fall into two principal groups: (1) the detection of the causative microorganism, *Treponema pallidum*, in lesions suspected of being syphilitic in nature, and (2) the detection of certain pathological changes in the body due to invasion by the organism.

Of the procedures for the detection of *Treponema pallidum*, the use of the darkfield microscope is most important, since it makes possible immediate demonstration of the organism in its natural state. Notwithstanding that considerable time has elapsed since Landsteiner and Mucha introduced dark ground illumination for the diagnosis of syphilis in 1906, few medical men understand the proper use of the darkfield microscope, and some have gone so far as to say that its employment furnishes a less reliable method for demonstration of *Treponema pallidum* than silver impregnation. This unfortunate situation is no doubt due to unskilled use of the darkfield microscope, hence the greater part of the chapter on microscopic technique has been devoted to description of the darkfield apparatus and directions for its manipulation.

Demonstration of the *pallidum* by a negative image impression is useful, provided that the background

is rendered uniformly homogeneous and dense, as in the method of Benians. Staining of the organism by various dyes, or by silver impregnation, is likewise important, and a number of the procedures are enumerated, including the new method of Miss Tilden, which makes it possible to stain the *pallidum* with the ordinary basic aniline dyes as well as by silver impregnation, or to examine them unstained but well preserved by darkfield illumination. The descriptions of the various staining methods have been incorporated, together with the discussion of darkfield microscopy, in Chapter XVI.

Of the biological or biochemical procedures for detecting indirectly the presence of a syphilitic infection, the most important is undoubtedly the Wassermann reaction. The chapters dealing with serological procedures cover the same ground as the "Serum Diagnosis of Syphilis," a third edition of which appeared in 1912, and have the same purpose, that of giving an elementary presentation of the principles of hemolysis and complement fixation as applied to the serum diagnosis of syphilis. During the decade which has intervened, certain modifications in technique have been proposed, but none has changed the fundamental procedures already in existence. The antisheep hemolytic system is still in general use, and the antihuman system has been adopted by Craig, Butler and Landon, and others,

and is now well established. That the latter, notwithstanding its being more rational and giving more reliable results, is less widely adopted is probably due to the alleged technical difficulties associated with the preparation of the antihuman hemolytic amboceptor. With regard to the varieties of the so-called syphilis antigens, no essential progress has been made since the introduction of the acetone-insoluble tissue lipoids. Bordet has recently recommended the same fraction as antigen. Some workers still use the plain alcoholic organ extracts with or without the addition of cholesterol.

Attempts are being made by various investigators to "standardize" the Wassermann reaction; yet no amount of accuracy in complement, antigen, and other factors, will be of any value so long as the amboceptor is not quantitatively adjustable. Errors due to the presence of an unknown and variable quantity of amboceptor are inherent in any hetero-hemolytic system.

During the past few years tests for syphilitic serum based upon the precipitation of certain protein fractions have been proposed—Bruck's nitric acid test, for example, and the precipitation test with lipoidal extracts with or without the addition of cholesterol, originated by Sachs and Georgi and modified by Meinicke, and by Dreyer and Ward. In the majority of cases they give results parallel with the

Wassermann reaction, but they sometimes give positive results in sera from diseases other than syphilis and negative results in syphilitic sera showing a strong Wassermann reaction. Inasmuch as the usefulness of these reactions as routine tests is not as yet established, I have not attempted to describe them in detail.

The various tests for increased protein or cellular content of the cerebrospinal fluid are essential in the diagnosis of syphilitic affections of the central nervous system. The butyric acid test, Amoss's phosphate solution test, and other methods, including Lange's colloidal gold reaction, are described, and a new procedure, based on the flocculation of lipoids, has been added.

The chapter on the luetin reaction has been considerably abbreviated. I have not changed my early view regarding its diagnostic value in certain chronic cases of syphilis in which the Wassermann reaction gives an indecisive result.

I wish to acknowledge my obligation to Dr. Paul A. Lewis, Dr. A. J. Rosanoff, and Dr. David J. Kaliski for their cooperation and suggestions in connection with the earlier editions, and to Miss Evelyn B. Tilden for her assistance in the revision of the present edition, especially the preparation of Chapter XVI. Thanks are also due to the publisher for his advice and courtesy in the preparation of this volume.

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CHAPTER I

SERUM HEMOLYSIS

THE red blood-corpuscles of animals when brought into contact with many different substances are so altered that their hemoglobin is set free, the stromata also going into solution as a rule. This phenomenon of solution is now generally known as *hemolysis*. The substances which cause hemolysis are said to be hemolytic for the blood-corpuscles which they dissolve. Fresh blood-serum of many animal species is hemolytic for the erythrocytes of some, but not all, other species. Hemolysis by serum results from the cooperative (coordinated) action of two distinct serum principles or factors. The first is called *amboceptor;*[1] the second, *complement.*[2] The latter is always present in all fresh sera; the former, on the other hand, is inconstantly so, frequently being absent from normal blood-serum. If erythrocytes are added to a serum which contains only amboceptor they absorb the amboceptor and

[1] Bordet's *substance sensibilisatrice*, and Metchnikoff's *fixateur*.

[2] Bordet's *aléxine*, and Metchnikoff's *cytase*.

retain it so firmly that even repeated washing with physiological salt solution cannot detach it from the corpuscles. The erythrocytes laden with amboceptor are said to have been *sensitized*. If complement be added to cells so prepared they promptly dissolve. Erythrocytes do not absorb complement from a serum if there is no amboceptor present. The function of the amboceptor is to prepare or *sensitize* the erythrocytes for the attack of the complement, and that of the complement is to *dissolve* the sensitized erythrocytes. Amboceptor alone cannot dissolve the cells, and complement likewise is ineffective if the cells are not prepared for its action. The particular constituent of the erythrocytes capable of uniting with the specific amboceptor is usually called the *receptor*.

A dilute suspension of erythrocytes in physiological salt solution presents a bright orange-red, opaque appearance. The cells may be sedimented to the bottom of the receptacle, either by centrifugation or by being allowed to stand for many hours, leaving above a clear, colorless fluid. After hemolysis, however, the suspension becomes deep pinkish red and transparent, being now a solution of hemoglobin diffused out of the hemolyzed erythrocytes (Fig. 1).

These two essential hemolytic components, amboceptor and complement, not only differ in their biological function, but also show differences in resistance to spontaneous deterioration, destruction by heat, and various other physical and chemical influ-

ences. Complement is labile and deteriorates gradually, disappearing from serum within a few weeks when kept on ice and within a few days when kept at room temperature. Exposure to a temperature of $55°$–$56°$ c. for one-half hour completely destroys the activity of complement. Amboceptor is much more stable. It is usually still active in serum which

FIG. 1. *a* shows a saline suspension of blood-corpuscles before hemolysis; *b*, the same after hemolysis. *a'* and *b'* present the appearance of *a* and *b* after sedimentation of corpuscles.

has been kept for more than a year, and is not destroyed or markedly injured by exposure to a temperature of $55°$–$56°$ c. Serum is technically known as "fresh" or "active" serum within a day

of its collection, while the complement is still fully active. The process of depriving a fresh serum of its complement by heating it to $55°$ c. is called *inactivation* of the serum. By an *inactivated serum* we mean one from which complement has been removed, but in which the amboceptor is left unchanged. If to an inactivated serum we add fresh serum (in a quantity inactive by itself because of its minuteness or the complete absence of amboceptor) the mixture may produce hemolysis in the presence of amboceptor in the inactive serum, because the complement destroyed by the process of inactivation is replaced by the complement of the fresh serum. This process of restoring the hemolytic activity of an inactivated serum by the addition of fresh serum is known as *reactivation*. Fresh serum thus used, inactive by itself, functions by virtue of its complement content, and is commonly called complement, being further specified by the name of the animal from which it is derived. Complement is always capable of reactivating the serum of the species from which it is derived, but not every complement can reactivate the sera of other species. In fact, there are only a few animals known whose complements can reactivate the inactivated sera of alien species, that is, complement of one animal species is not identical in its action with that of another species. The interchangeability of complements, or

the substitution of one for that of another species of animal, is only possible in a limited number of instances. The complement of the guinea-pig is distinguished by an unusual ability to reactivate the sera of alien species and is consequently most often used when it is necessary to substitute the complement of one serum for that of another which has been inactivated or which has deteriorated.

The presence in blood-serum of natural amboceptor for erythrocytes of alien species is much less constant than that of complement.

The amboceptor of any species acts always with the complement of the same species and less regularly, and to a limited extent only, with the complement of other species. In its relation to the red blood-corpuscles, however, the amboceptor is *specific:* that is, an amboceptor which can sensitize the erythrocytes of the rabbit, for example, to the action of complement cannot sensitize the erythrocytes of the sheep, dog, or any other animal. Amboceptors are named by prefixing "anti-" to the species-name of the cells against which they act. For example, an amboceptor active against sheep-corpuscles is known as antisheep amboceptor and unites with the receptors of the former.

In any serum there are usually present many varieties of amboceptor. For example, in one serum there may be found amboceptors active against the

blood-corpuscles of the sheep, dog, hen, rabbit, frog, man, etc. Different sera vary widely in the number of amboceptors present and in the relative quantity of each. Among different specimens of serum from the same species the relative quantity of amboceptor may also vary considerably.

Amboceptors existing naturally in normal serum are known as *natural* or *normal amboceptors*. For example, human serum is frequently, though not always, quite hemolytic for sheep's corpuscles, because it may contain natural antisheep amboceptor; but rabbit's serum, on the other hand, is incapable of hemolyzing human erythrocytes because of the absence in the rabbit of natural antihuman amboceptor.

As already stated, the serum of a given species may not contain amboceptors for the blood-corpuscles of some other species. If we select, for example, the rabbit, whose blood-serum contains no amboceptor for the erythrocytes of man, we may by repeated injections of the human erythrocytes into this animal produce specific amboceptor for human cells. This process of repeated injections with foreign cells (or any other suitable substance) is, in general, known as *immunization*. By a similar process we can also *increase* the amount of an amboceptor naturally present. Amboceptors thus artificially produced or increased are known as *immune amboceptors*. Amboceptors which act with the erythrocytes of the same

species are known as *isohemolytic amboceptors*, or, in short, *isohemolysins*. It is extremely difficult to produce an amboceptor for the erythrocytes of the same species by immunization.

The amount of complement is not perceptibly increased by immunization.

Summary. It has been found that the fresh serum of an animal can hemolyze the erythrocytes of as many species as its serum contains specific amboceptors for, the intensity of hemolysis being proportional to the amount of a given amboceptor present in the serum. The variety of natural amboceptors varies considerably in the different animal species.

The hemolysis is caused by the specific, coordinated interaction of the amboceptor and complement of the serum on the erythrocytes. Complement is destroyed by heating to 55°–56° c. for half an hour, the serum being thereby inactivated. Amboceptor is not destroyed by this process. The hemolytic activity of the serum can be restored by replacing the destroyed complement by complement contained in fresh serum of the same species or in that of a limited range of alien species whose complements are suitable for use. By immunization we can create a specific amboceptor for any kind of foreign erythrocytes. This immunization product possesses apparently the same biological properties as the natural amboceptor and is called *immune amboceptor*.

CHAPTER II

QUANTITATIVE FACTS ABOUT HEMOLYSIS

THE phenomenon of hemolysis, as pointed out in the previous chapter, is dependent upon the action of complement and amboceptor upon erythrocytes. The first is contained in every fresh serum, the second may be contained in a given serum, or it can be artificially produced by immunization.

The hemolytic activity of any serum is usually determined as follows. A uniform quantity of a suspension of red corpuscles (erythrocytes) in a physiological salt solution[1] is mixed, in a series of test-tubes, with graduated amounts of the serum and the whole brought to a constant volume by the addition of salt solution. The tubes are placed at a temperature of 37° c. for a sufficiently long time to allow a complete reaction, usually for thirty minutes in a water bath or one hour in an air thermostat. The amboceptor *titre* of the serum is usually expressed by the smallest amount of serum which is found to be necessary for the complete dissolution of all the corpuscles. This method of titration is usually applicable, because in ordinary normal serum, complement is often present in excess of the amount needed to activate all the amboceptor contained in the

[1] For hemolytic work 0.85 per cent (Ehrlich) to 0.9 per cent (Madsen) salt solution is generally employed. The writer uses the latter concentration.

serum. In the case of normal serum rich in natural amboceptor or an immune serum, in both of which the normal complement content is associated with a great excess of amboceptor, a titration by the method of simple dilution would disclose only the amount of complement in the serum, and a large, variable amount of amboceptor would remain inactive in the mixture, because of the dilution of complement to a minimum. In order to arrive at the value of the serum in terms of its amboceptor another procedure must be adopted. A definite amount of corpuscle suspension is placed in each of a series of test-tubes, as before, and to each tube is then added an amount, also definite and equal, of a normal serum which has been found incapable in itself of causing hemolysis (pp. 6, 7). There are next added, in series, decreasing, graduated amounts of the serum for titration whose native complement has been previously destroyed by inactivation. The amboceptor titre of a serum is the smallest amount of inactivated serum which produces complete hemolysis in the presence of a sufficient amount of a given suitable complement. It must be borne in mind that the titre of an immune serum will vary with the specific activity of the complement used. For example, an antihuman amboceptor prepared by immunizing a rabbit with human corpuscles is more active in the presence of a given amount of guinea-pig serum used as comple-

ment than it is when tested with the same quantity of human serum as complement. The strength of the immune serum, expressed in terms of the smallest amount needed to produce complete hemolysis, will be quite different if 0.1 c.c. of complement has been used, than if 0.05 c.c. has been used. *Within certain limits, the quantitative relationship existing between the absolute amount of complement and amboceptor required to produce complete hemolysis is such that an increase of one factor, say complement, permits the use of a smaller amount of the other factor, namely, amboceptor.*

In order to get uniform results with a reaction in which so many of the reagents are variable in activity, it is necessary to proceed in a definite order to fix standards of practical constancy. This order has been roughly outlined but will bear restatement. A suitable suspension of erythrocytes is chosen and the amount of this suspension to be used arbitrarily fixed and kept constant. The total volume of the mixture is decided upon as another constant. A moderately large and uniform amount of complement is used in the first determination, an amount certainly in excess of that required for the complete activation of the minimal amount of amboceptor. With this amount of complement, in series, are combined decreasing quantities of amboceptor. The smallest amount of amboceptor required to produce complete hemolysis

is determined; this is not only, as has been already stated, the amboceptor titre or value of the immune serum, but it is best chosen for future work as the second fixed value in the reaction, the erythrocyte suspension being the first. In order to be sure that the amboceptor titre is a constant value, another test should now be made in which the quantity of amboceptor is still further reduced while the complement quantity is doubled. If all the tubes in this series are not completely hemolyzed we may be certain that the complement quantity first chosen was large enough and that the amboceptor amount determined was actually the least amount which could under any circumstances produce complete hemolysis of the corpuscle suspension used. This amount of amboceptor may be conveniently designated as one *amboceptor unit*.

Up to this point the amount of complement has been kept in excess. Now to each of a series of tubes containing the standard erythrocyte suspension one unit of amboceptor is added. If then in the series one puts decreasing amounts of complement the smallest amount of complement necessary to produce complete hemolysis with one amboceptor unit will be determined. This amount is called one *complement unit*. The reaction could then be formulated as follows:

Standard erythrocyte suspension + 1 amboceptor

unit + 1 complement unit = complete hemolysis
(incubated for thirty minutes in the water bath or
one hour in the thermostat at 37° c.).

As the erythrocyte suspension can be made of
relatively constant value from day to day, and as the
amboceptor is stable over a period of months, the
only actual variable from this point is the comple-
ment, and this can be standardized with a simple
titration test.

The results, now, of varying the quantities of com-
plement and amboceptor require the most careful
consideration. If less than one unit of amboceptor
is used hemolysis will always be incomplete, even
with more than one unit of complement. Likewise,
if with one amboceptor unit there is combined less
than one unit of complement, hemolysis cannot be
complete. If with more than one unit of amboceptor
there is used less than one unit of complement,
hemolysis may be complete or incomplete according
to the relative amounts of each factor used. The
hemolysis is of the same degree whether produced
by a given amount of amboceptor plus just enough
complement to complete hemolysis, or by 20 times
that amount of amboceptor with 1/10 as much com-
plement, or by any other appropriate combinations
of these two components. *Unless the amount of ambo-
ceptor used is the same in any two sets of hemolytic
experiments, the amount of complement acting in*

TABLE I

AMOUNT OF ANTISHEEP AMBOCEPTOR (FROM IMMUNIZED RABBITS)

Aml. of complement (guinea-pig)	Series 1 0.00004	Series 2 0.00006	Series 3 0.00012	Series 4 0.00024	Series 5 0.0003	Series 6 0.00042	Series 7 0.0006	Series 8 0.0012	Series 9 0.0024	Series 10 0.0048
0.1	90 %									
0.08	50 %	100 %								
0.06	30 %	70 %	100 %							
0.05	20 %	50 %	90 %							
0.04	10 %	30 %	40 %	100 %						
0.03	0	10 %	20 %	90 %						
0.025		5 %	10 %	70 %						
0.02		0	0	20 %	100 %					100 %
0.015				5 %	90 %	100 %			100 %	90 %
0.01				0	50 %	80 %	100 %	100 %	80 %	50 %
0.007					20 %	40 %	50 %	50 %	40 %	10 %
0.005					0	10 %	20 %	20 %	10 %	0
0.004						0	0	0	0	
0										
No. of amboceptor units in each series........	⅔	1	2	4	5	7	10	20	40	80
Complement necessary for 100 % hemolysis.........	> 0.1	0.1	0.06	0.03	0.02	0.015	0.01	0.01	0.015	0.02
For 0 % hemolysis........	0.03	0.025	0.02	0.007	0.005	0.004	0.004	0.004	0.004	0.005
Activity constant of complement for 100 % hemolysis..	< 1	1	1.66	3.33	5	6.66	10	10	6.66	5
For 0 % hemolysis........	0.8	1	1.25	3.57	5	6.25	6.25	6.25	6.25	5

these two sets cannot be estimated by comparing the degree of hemolysis. In order to calculate the exact amount of complement needed to produce a given amount of hemolysis, it is necessary to know the amount of amboceptor used, because the activity of complement is different according to the amount of amboceptor present. It is therefore erroneous to conclude that an equal degree of hemolysis, produced by 1 unit of amboceptor and by 20 units of amboceptor, is the work of the same amount of complement in both instances. These facts are graphically shown in the accompanying diagrams (Figs. 2, 3 and 4). In Table 1 are given the quantitative data of an experiment to determine the relative amounts of complement and amboceptor necessary to produce a given degree of hemolysis.

For any given relative proportion the result can be determined experimentally, of course, and the reaction follows certain laws with sufficient regularity so that the result can be calculated. But it is exactly for the purpose of avoiding the complexities and uncertainties introduced by this quantitative variation that we take such pains to get fixed standards, and the more closely these standards are adhered to the simpler will be the conditions and the easier and more accurate the interpretation of the results.

We shall now consider the fate of amboceptor and

Green = Complement
Purple = Amboceptor
Red = Haemolysis

20 units of Amboceptor used in each combination
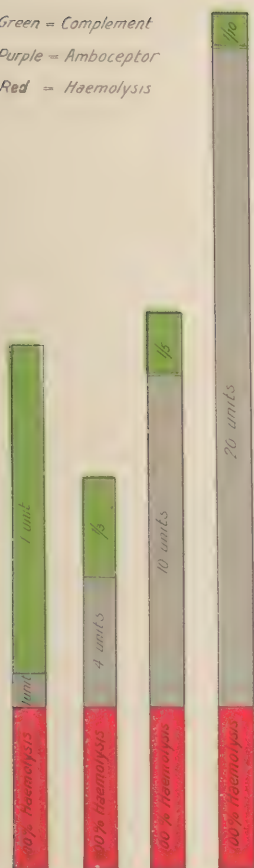
1 unit of Amboceptor used in each with various fractions of a complement unit

Fig. 2.

Fig. 3.
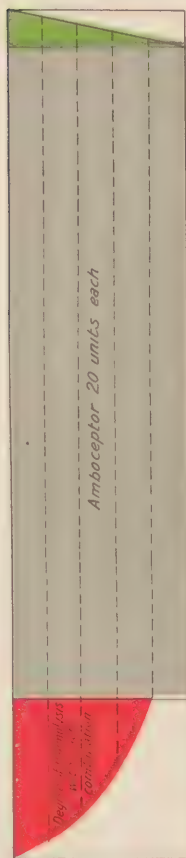
Fig. 4.

complement after the reaction has proceeded to completion. It has been experimentally determined that within certain quantitative limits they have disappeared from the mixture, having been exhausted in producing hemolysis. The limits within which this disappearance is complete are roughly as follows: When one unit of amboceptor has been used with one unit of complement the disappearance of both will be complete. When with one unit of amboceptor more than one unit of complement is used, an excess of complement will be found still present in the fluid after the reaction is accomplished. The same applies to the combination of one unit of complement with less than one unit of amboceptor, in which case hemolysis will be incomplete. When with one unit of complement there is combined more than one unit of amboceptor the excess of amboceptor shortens the time necessary for complete hemolysis. The corpuscles, being capable of absorbing more amboceptor than is necessary for complete hemolysis, take up most of the excess in this way. If the excess is very large (more than the corpuscle mass can absorb), it may be found free in the fluid after the reaction is ended. If more than one unit of amboceptor and of complement are used, an excess of both may be found in the fluid after hemolysis is complete.

Enough has been said to show that we are dealing with a complex reaction whose factors have a definite

relationship to one another which can be accurately determined. If the reciprocal values of complement and amboceptor are carefully determined and rigidly adhered to according to the outline given, precise and constant results can be obtained in the practical application of the reaction, which will be developed in the following pages. If in using the reaction these quantitative steps are not observed, one cannot hope for useful or accurate results.

CHAPTER III

ANTIGENS AND ANTIBODIES

IT has already been stated that by repeated injections of erythrocytes of one animal into an alien species we can produce in the latter an amboceptor having a specific affinity for these erythrocytes. A similar phenomenon is observed when bacteria are injected. We call amboceptors for the blood-corpuscles *hemolytic amboceptors* and those for the bacteria *bacteriolytic amboceptors*. Their general characteristics are the same; they differ only in that they have a specific affinity towards the substances which gave rise to them. Bacteria when brought into contact with specific bacteriolytic amboceptor absorb the latter and become sensitive to the dissolving action of complement. This phenomenon of dissolution is called *bacteriolysis* and in its mechanism is comparable to that of *hemolysis* in every respect.

It is found that when various unorganized protein substances are injected into an animal they elicit a similar response, giving rise to various specific immune substances or reaction products. The injection of egg albumin and alien blood-serum, for example, gives rise to specific *precipitins*. When a serum containing a specific precipitin is mixed with a solution of the protein which was injected to develop it, a precipitate is formed, but if the serum is mixed

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with any other protein solution the precipitation does not occur. The substances—erythrocytes, bacteria, or unorganized foreign protein—which when injected produce corresponding specific reaction products are called *antigens.* Reaction products of any kind whatever which are produced by the animal are called *antibodies.* Immune hemolytic amboceptors, bacteriolytic amboceptors, and precipitins are therefore antibodies produced by injecting as antigens erythrocytes, bacteria, or unorganized proteins.

The most striking characteristic of the antibodies is their specific relation to the corresponding antigens. Antigen A gives rise to antibody A, and antibody A reacts outside the body with antigen A and with no other. It is scarcely necessary to recall the well-known fact that the serum of a typhoid patient (containing typhoid agglutinin) agglutinates only the typhoid bacillus and none of the closely related intestinal micro-organisms. The hemolytic amboceptor for human erythrocytes acts only with those erythrocytes and not with the cells of any other species. Similarly, as has been stated, the precipitin obtained by injecting serum of man, monkey, or rabbit into alien species causes precipitation only with the particular serum used to produce it. The few exceptions to this general rule may be disregarded in our present discussion.[1]

[1] For example, I found that antigoat serum (rabbit) contains precipi-

This quality of specificity having been taken into account, it is evident that if we have an unknown antibody to deal with we can identify it by putting it in contact with a number of different antigens under favorable conditions and noting the one with which it reacts. With a known antibody the character of an unknown antigen can likewise be determined. This *direct method* of recognizing unknown antibody has been used in a number of different ways. Some instances, such as the Widal reaction in typhoid fever, are known to every one. Another important test dependent on this principle is the precipitation method for determining the species of animal from which a specimen of blood of unknown origin may have come. Artificial antibodies are produced by immunizing animals—rabbits, for example—with the blood-serum of a number of different animal species. The unknown blood is dissolved in physiological salt solution and brought into contact with this series of known antibodies (precipitins). That antibody with which a precipitate is formed must be, according to the law of specificity, antibody prepared with serum of the same species as that from which the specimen in question was derived.

tins not only for goat serum but also for sheep and ox sera in smaller quantity, while anti-ox serum (rabbit) contains precipitin only for ox serum. Antisheep serum contains precipitin for sheep and goat sera but not for ox serum. This phenomenon is comparable to the group reaction of agglutination.

The effects we have so far considered have all been the direct and essential manifestation of reactions between antigen and antibody with or without the associated action of complement as the case may be.

In studying the phenomena of interaction of antigen and antibody in general, we shall discover a peculiar relation which exists between the combination of antigen-antibody and complement. We have already seen that the erythrocytes (antigen) acted on by amboceptor (antibody) become so altered as to absorb complement and undergo hemolysis. We have also learned that bacteria (antigen), after having been acted on by amboceptor (antibody), take up complement and become dissolved by the complement. Now a question arises as to whether unorganized antigens display the same characteristics as the antigens in previous instances when brought together with their specific antibodies. It has been found by experiment that they do. For example, when a precipitable antigen (precipitinogen) is brought into contact with its specific precipitin it not only forms a visible precipitate but also becomes capable of absorbing or fixing complement. If to a mixture of a precipitable antigen—for example, blood-serum or egg albumin—and its precipitin, complement be added during or after the reaction period, and if the mixture be subsequently tested for the presence of complement by adding erythrocytes and their specific hemolytic am-

boceptor to the mixture, it is found that the complement has disappeared; that is, hemolysis does not take place. This phenomenon of disappearance of complement in the mixture of antigen and antibody is now generally called *fixation of complement*. Sometimes it is called *deviation of complement* because of the fact that the complement has been deviated by the combination of antigen and antibody and prevented from participating in the hemolytic process. These facts were first brought out by the investigations of Bordet and Gengou, and the reaction is known accordingly as the Bordet-Gengou phenomenon of complement fixation.

It is found that the mixture of antigen and antibody can fix complement in a dilution in which a visible precipitation is no more obtainable. In other words, the fixation phenomenon is capable of indicating the existence of the antigen-antibody reaction with a delicacy beyond that which visible precipitation can attain.

In connection with the phenomenon of complement fixation, it would be well to point out that the sera (complement) of various animals present marked differences in their ability to be fixed. Some complements are easily fixed in the presence of the antigen-antibody combination, others slightly or not at all. While the serum of an animal may possess the property of reactivating the hemolytic amboceptor of

FIG. 5. Shows the components required and the steps of reactions followed in producing hemolysis, bacteriolysis, and adsorption or fixation of complement by a precipitate.

Fig. 6. Illustrating the phenomenon of Bordet and Gengou. The serum reaction of Wassermann for syphilis is analogous to Example 3, except that the lipoidal substance corresponds with protein and syphilitic serum with precipitin.

an inactivated serum, yet the serum of this species may possess little or no fixation property. This fact becomes of great importance, as will be seen later, in the utilization of the complement-fixation phenomenon in diagnosis.

Working with three different antigen-antibody combinations, namely, precipitates formed by mixing human serum, egg albumin, and meningococcus extract with their specific precipitins, the writer has found that the fixability of the sera (complement) of various animals differs widely. Human complement was readily fixed, that of the guinea-pig most easily and goats' complement hardly at all. The complements of the horse, ox, sheep, and rabbit varied in fixability in degrees intermediate to these extremes. In performing these experiments two different amboceptors (both specific for human corpuscles) produced in rabbits and goats were used. The complements of the rabbit and guinea-pig possessed, as a rule, the best reactivating property for the rabbits' amboceptor; those of the goat and the horse were less efficient, and the complements of sheep, pig, and ox were far inferior and were often devoid of reactivating power. The amboceptor from goats could be reactivated by all of the sera mentioned excepting that of pigs, although goats' serum was most efficient and guinea-pigs' somewhat less so. Human complement was quite active with these amboceptors.

We have also seen the mode of detecting, by direct observation of the precipitation reaction, an unknown antigen or antibody. Now antigen can also be detected, by indirect observation, through the employment of the complement-fixation reaction. To illustrate the mechanism of the Bordet-Gengou phenomena (pp. 22, 23) schematic illustrations are introduced based upon the well-known side-chain theory of Ehrlich. Fig. 5 shows three different combinations of antigens and antibodies, each capable of absorbing or fixing complement. Fig. 6 illustrates the deviation of complement by one combination of antigen and antibody, which prevents the complement from taking part in a subsequent reaction of the same kind.

The application of this indirect method of determining the presence of a specific reaction between antigen and antibody has been extensively applied to various infectious diseases with more or less success. The details of the application of this principle to syphilis will be developed in the following pages.

CHAPTER IV

The Application to the Diagnosis of Syphilis of the Indirect Method of Determining Antibodies

WE have so far discussed the fact that combinations of antigen and antibody which do not require complements to complete their characteristic reaction may still bind complement and prevent its taking part in other reactions. We have also noted that the property of fixing complement may be exerted by quantities of antigen and antibody which are too small to give rise to the characteristic reaction of such a combination, namely, visible precipitation. From this point it was but a short step to the conception that there might be a combination of antigen and antibody with no characteristic direct manifestation with which we are acquainted but one which could still exert a fixing effect on complement.

From clinical studies it has long been known that syphilis is an infectious disease which in running its course may produce a specific immunity. That an immunity is developed means, presumably, that antibodies against the infectious agent are produced in the subject at some stage of the process. Moreover, the detection of the presence of *Treponema pallidum* is not to be relied upon as our only means of diagnosis. In the late manifestations of the disease, at a

time when it is still active and still amenable to specific treatment, the treponemata are present in such small numbers as to be most difficult of detection. In exactly these cases a measure of immunity may be supposed to have developed and specific antibodies to have been formed.

The idea, then, was to take syphilitic tissues at a stage when the treponemata were most numerous and use this as the known antigen. Tests against this known antigen with blood-serum of other syphilitic patients might, it was hoped, reveal the presence of antibody. The direct methods of observation such as agglutination or bacteriolysis having failed, the indirect method was tried.

The earliest publication on this indirect method of detecting the syphilitic antibody is that by Wassermann, Neisser and Bruck on May 10, 1906, and the next article is that by Ladislaus Detre on May 24th of the same year. These investigators were working on the same subject independently during the same period of time and obtained exactly the same results. The technique employed by Wassermann, Neisser and Bruck, on the one hand, and by Detre, on the other, was identical except in small details.

Extracts of syphilitic tissues in the active stages of the disease were used as antigen. Wassermann, Neisser and Bruck used the liver of a congenitally syphilitic fetus, and Detre employed chiefly condyl-

omata for this purpose. Serum of known syphilitics, inactivated at 56° c., was used as antibody. To this combination complement was added. Up to this point there was no visible change. If after a short time a quantity of immune hemolytic amboceptor was added to the mixture, and then the cells for which this amboceptor was developed, no hemolysis took place. It can be shown by suitable experiments that the failure of the erythrocytes to dissolve is not due to any change in the cells, nor is it due to interference with the amboceptor. The complement has been prevented from acting, has been fixed, or deviated. If in place of the blood-serum of a known syphilitic there was used the blood-serum of a person known never to have had syphilis, hemolysis occurred, because the complement was not interfered with. The erythrocytes used by Wassermann, Neisser and Bruck were those of the sheep, and those used by Detre were from the horse. The amboceptors employed were an antisheep in the first instance and an antihorse in the second, both being derived from rabbits immunized with the erythrocytes of the respective animals.

In the beginning it was supposed that the reaction was specific; that is, that the serum of the syphilitic patient would only fix complement in the presence of extracts of syphilitic tissues. The experiments of Landsteiner, Müller and Pötzl, and later of Leva-

diti, Weil and Braun, Meier, and also of the writer, have shown that when such a serum is combined with the alcoholic extracts of certain normal organs (heart or liver) or with a preparation of tissue lipoids (phosphatids) from the heart or liver, complement is also fixed. Because they are easier to obtain and yield results entirely identical with those from syphilitic tissues such extracts, sometimes reinforced with cholesterin, are now commonly used as antigens in the complement fixation reaction. When the diseased tissues are used as the source of antigen the liver of a syphilitic fetus is commonly chosen.

Summary. It has been found on the basis of thousands of comparative tests that if the blood-serum of a patient suffering from syphilis be mixed in the presence of complement with extracts of syphilitic livers, with alcoholic extracts of certain normal organs, or with preparations of tissue lipoids, the complement will be fixed and prevented from taking part in a subsequent hemolytic reaction. Therefore, when the extracts given are mixed, in the presence of complement, with serum of unknown origin, if complement be fixed it can be stated with assurance (with certain reservations to be discussed later) that the unknown serum was derived from a case of syphilis.

To those who have carefully considered the quantitative relationship of the factors in a simple reaction

with a hemolytic serum as presented in the first chapters of this book, it will at once be apparent that in a test in which the hemolytic reaction is to be used as an indicator of a second reaction not otherwise visible, the quantitative values of all the factors must be accurately determined to insure reliable results. The quantitative relationship of these factors will now be considered.

QUANTITATIVE RELATIONS OF THE FACTORS IN THE SERUM DIAGNOSIS OF SYPHILIS

THE five distinct and essential factors which enter into operation in the complement fixation reaction may be enumerated as follows: The so-called syphilitic antigen and syphilitic antibody, red blood corpuscles, hemolytic amboceptor and complement. To avoid confusion in the discussion of the reaction it has been customary to group these factors according to their functions as follows: the corpuscles, the hemolytic amboceptor, and the complement are collectively referred to as the *hemolytic system*. Speaking of the first two factors of the hemolytic system it is customary to avoid the terms antigen and antibody and speak of corpuscles and amboceptor. When in connection with the test we refer to antigen and antibody, we mean only the two factors outside the hemolytic system, the so-called syphilitic antigen and the antibody present or absent in the serum to be tested. In principle any hemolytic system can be used as an indicator for the test, provided the complement used is sensitive to fixation.

Whatever system be chosen, the relation of amboceptor, complement, and corpuscles as outlined in the second chapter must be observed; that is, one must work with a suspension of corpuscles of

definite value, the amboceptor must be carefully titrated with respect to that corpuscular suspension, and with respect to the complement employed. Definite amounts of amboceptor must be used, and the quantity of complement to be employed must be constant and so adjusted as to act with the quantity of amboceptor and cell suspension determined upon. If, for example, we use in the hemolytic system one amboceptor unit, at least one complement unit must be combined with it in order to obtain complete hemolysis. If less than one complement unit were added to the antigen-antibody mixture in the fixation test, hemolysis would certainly be incomplete, and one might imagine that complement was fixed when it was merely deficient in quantity from the beginning. If many amboceptor units are used hemolysis may be complete in the presence of much less than one unit of complement. Theoretically the test for fixation of complement might be made much more delicate by uniting a maximum amount of amboceptor with the minimum amount of complement that can produce a complete hemolysis. In practice, however, there are certain extrinsic factors which may interfere with the action of small amounts of complement, hence it is not safe to use minimal quantities. These factors will be more carefully and fully considered later.

A common quantitative error is the following:

Suppose that the antigen-antibody combination in any given test is capable of binding one and one-half units of complement. The one-half unit of complement remaining, two units being used in the test, would be incapable of completing hemolysis with the unit amount of amboceptor, but with a high multiple of that amount would produce complete hemolysis. A partial fixation of complement would then be obscured by an excess of amboceptor. If, on the other hand, with a small amount of syphilitic antigen and antibody we use an excessive amount of complement, the antigen-antibody combination may bind complement up to its capacity and still leave a sufficient quantity to act in conjunction with the amboceptor to produce hemolysis, that is, we should fail to detect the fixation of complement actually occurring.

It has been stated that certain factors not directly involved in the reaction might interfere with it if small amounts of complement are used. There are at times substances in the extracts other than the one functioning as antigen which prevent complement from acting. They are commonly known as anticomplementary substances, and they may also exist in the serum to be tested. In order to guard against error from this source, serum and antigen must be tested for the presence of anticomplementary substances by adding each singly to the stan-

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dard hemolytic system in somewhat larger quantity than they are to be used in the test.

On the other hand, human serum may contain natural hemolytic amboceptors for the erythrocytes in use when the erythrocytes come from alien species. For example, if we add one unit of *immune* amboceptor to a serum already containing six units of *natural* amboceptor, it would produce exactly the same result as though seven units of the immune amboceptor had been added. This source of error can be avoided by choosing a hemolytic system for which human serum contains no natural hemolytic amboceptors.

There may also be hemolytic substances in the antigen preparations; hence when unknown or freshly prepared extracts are used they must be tested for these substances.

Complements of different species of animals behave differently toward the fixation by antigen-antibody combinations. Certain species of animals contain in their sera complements which are readily fixed, while the complements of other species are quite refractory or not at all susceptible to the fixation. It is generally conceded that the complement contained in guinea-pig's serum is the best for the fixation tests.

With regard to the exact quantities to be used, the determination of the strength of amboceptor and

complement has been described in detail (pp. 11–16). It is customary to use a slight excess of each, usually two units. This makes the reaction somewhat less delicate but allows a margin for error due to anti-complementary substances and in the long run makes the test more reliable. The amount of serum to be tested for its antibody content must be large enough to bind all complement in the presence of sufficient antigen if the serum be from a known syphilitic case. *The proper quantity of the serum to be used depends, therefore, upon the quantity of the complement used in the hemolytic system.* It is obviously of advantage to construct a system in which as small an amount as possible of patient's serum can be employed without diminishing the delicacy or reliability of the reaction.

CHAPTER VI

Various Forms of the Complement-fixation Test as Applied to the Serum Diagnosis of Syphilis

A S pointed out in the foregoing chapter, it is erroneous to think that almost any hemolytic system can be used to test the binding power of antigen-antibody combination for complement. On the contrary the serologist must take into consideration the disturbing effect that results from the use of a hemolytic system in which alien erythrocytes naturally susceptible to the hemolytic action of human serum are employed. In this case the amount of the amboceptor becomes uncontrollable and extremely variable, for the reason that the amounts of the natural amboceptor contained in the serum to be examined are unknown and variable. The second important point which the serologist must always bear in mind when employing a hemolytic system is the quality of complement as regards the fixation phenomenon. As stated in the foregoing chapter, different complements vary considerably in their activity and fixability. The selection of the hemolytic system is, therefore, one of the most important problems involved in complement-fixation tests and demands the utmost care and consideration on the part of the serologist. The importance of the hemo-

lytic system was not at first appreciated, owing chiefly to the lack of exact experimental knowledge, and various hemolytic systems were indiscriminately proposed. It is not difficult to determine the merit of these methods; some are such as to permit a quantitative determination of the reaction, others are too inaccurate for quantitative use. They may be divided into two groups according as foreign or human corpuscles are used in the hemolytic system. A brief critical review of these will perhaps make clearer the principles of the test.

METHODS IN WHICH FOREIGN ERYTHROCYTES
ARE USED

Wassermann, Neisser and Bruck used sheep blood-corpuscles, an immune hemolytic amboceptor made by immunizing a rabbit with sheep's erythrocytes, and guinea-pig complement. In making the test the syphilitic serum is inactivated. The quantity used is 0.1 c.c. and 0.2 c.c. for each specimen of serum. Two units of the amboceptor and 0.1 c.c. of guinea-pig's complement are used against 1.0 c.c. of a 5 per cent suspension of the washed sheep-corpuscles. The resultant volume of the whole mixture is brought up uniformly to 5 c.c. There is but one source of error, that is, the variable amount of natural antisheep amboceptor existing in human serum. When such an amboceptor is present in appreciable quantity it

increases the total effective amboceptor in the mixture and, in accordance with the relation existing between the amount of amboceptor and complement respectively required for complete hemolysis, tends always to make hemolysis complete even when antigen-antibody has fixed a considerable amount of the complement (Figs. 2, 3, 4, Table I, and pp. 12–16). It is also possible that the complement which has been completely fixed by a moderate amount of antigen-antibody combination may again be detached, if the amount of the hemolytic amboceptor introduced be very large, and produce complete hemolysis. The following table shows how often and in what amounts the natural antisheep amboceptor is encountered in human serum. In two specimens 60 amboceptor units were found in 0.2 c.c. of inactivated serum.

TABLE II

TITRATION OF NATURAL ANTISHEEP AMBOCEPTOR IN HUMAN SERUM

Sera	No. of specimens examined	No. of units of antisheep amboceptor in 0.2 c.c. of human serum ($56°$ C.) titrated with guinea-pig complement										
		None	<1	1	2	3	4	5	6	7	8	>10
Syphilitic sera......	190	19	21	42	21	25	26	17	8	4	3	4
Non-syphilitic sera..	111	3	15	25	31	10	12	8	5	1		1
Normal sera........	25	1	3	5	4	3	1	2	2	3	1	
Total..............	326	23	39	72	56	38	39	27	15	8	4	5

The disturbing effects of an excess of amboceptor in actual determination of syphilitic antibody in the

Wassermann reaction can be shown in the following table:

TABLE III

REVERSION OF REACTION THROUGH EXCESS OF AMBOCEPTOR

Wassermann Antisheep System

No. of units of amboceptor	Syphilitic anti-body 1 unit	Syphilitic anti-body 3 units	Syphilitic anti-body 10 units
1	++++	++++	++++
2	++++	++++	++++
3	++	+++	++++
5	−	+	++++
10	−	−	++++
20	−	−	+++
40	−	−	+

As the occurrence of hemolysis means a negative reaction, or absence of syphilitic antibody, the error in this case is always in the direction of throwing into the negative class sera with smaller amounts of syphilitic antibody. If an error in diagnosis is inevitable it is of course safer to have it in this direction; but, as will be pointed out later, this source of error can be avoided by employing an antihuman hemolytic system.

Apprehension has been expressed by some that human serum may contain sufficient hemolysin for human cells of Moss's Group 1 class to reverse a positive reaction in the antihuman hemolytic system, but the writer has not been able to confirm this finding and is still unconvinced that 0.1 c.c. of serum contains sufficient isohemolysin to interfere with

the diagnosis of syphilis by the antihuman method. In a series of 155 human sera titrated for their hemolytic action against 0.1 c.c. of a 10 per cent suspension of human corpuscles of Group 1, only one specimen was found which contained sufficient isohemolysin in 0.1 c.c. to bring about complete hemolysis. Two other sera gave 80 per cent hemolysis, one 50 per cent hemolysis, one 20 per cent, and three a slight trace. On the other hand, 93 sera of the same series contained sufficient antisheep amboceptor to cause complete hemolysis; in 48 of these hemolysis was complete in from two to seven minutes, in 26 within eight to twelve minutes, in 7 within fifteen minutes, and in 12 within twenty to thirty minutes. Of the remaining 62 sera only 9 were altogether devoid of hemolytic action on sheeps' cells, others gave slight hemolysis, and the remainder (44) varying degrees of hemolysis.

Bauer, in his modification of the Wassermann system, relies entirely for amboceptor upon the natural antisheep amboceptor in human serum, which, as has been pointed out, is a source of error in Wassermann's system. This procedure does not eliminate the error, however, because amboceptor is not always present in sufficient quantities and when present varies greatly in quantity, and is sometimes in great excess.

Hecht relies not only on the natural antisheep amboceptor of human serum but also on human com-

plement. If this were a system whose factors were regular in quantity, it would be much simpler in practice than Wassermann's system. It would only be necessary to add antigen to the patient's own serum, which would contain complement, and antibody to be detected, and amboceptor. After a period of incubation sheep erythrocytes would be added and after another period of incubation the test read. However, not only is the amboceptor a factor varying from zero to 20 units or more, but human complement is less regular in activity than is guinea-pig's complement. The test must be done with fresh serum, otherwise the complement would surely be reduced or totally lost by spontaneous deterioration.

Weinberg has modified Hecht's technique by titrating the fresh human serum for its hemolytic activity against sheep cells, using a uniform quantity of the patient's serum with increasing amounts of a 5 per cent suspension of sheep's cells. A series of 10 tubes is set up, and the series number of the tube in which complete hemolysis occurs is called the hemolytic index. Gradwohl employs Weinberg's method of titrating the hemolytic activity of the serum, but the amount of cell suspension which he uses in the actual test is about one-fourth or one-fifth of that which the specimen can hemolyze, that is, if the serum hemolyzes 0.1, 0.2, 0.3, or 0.4 c.c. of the cell suspension, 0.1 c.c. is used in the test.

Complete hemolysis is thus assured in the test in which antigen is added. Three different amounts of antigen are employed, 0.1, 0.15, and 0.2 c.c.

The number of tubes required for each specimen is inconveniently large when a large number of tests have to be made. The chief drawback of the method, however, is that the titration does not indicate the exact amounts of complement or amboceptor involved, but only their joint activity. In other words, an unknown quantity of complement is being used in the test for titrating by complement fixation the amount of syphilitic antibody present, since the amount of complement fixed is proportional to the amount of syphilitic antibody. As has already been said (pp. 8–16), 2 units of complement, in combination with 2 units of amboceptor, will be fixed by 1 unit of syphilitic antibody; 1 unit of complement in combination with 2 units of amboceptor will be easily fixed, even by half the amount of syphilitic antibody; on the other hand, if 10 complement units are combined with only 1 unit of amboceptor, then ten times as much syphilitic antibody will be required for complete fixation.

Stern proposed a system in which a few units of immune antisheep amboceptor were added to the fresh serum to be tested, the complement of the patient's serum being utilized. This procedure retains all the defects inherent in the use of an un-

known and often excessive amount of the hemolytic amboceptor, and makes it impossible to test a specimen that has been kept a few days after collection.[1]

Detre and Brezovsky used horse corpuscles, an immune antihorse hemolytic amboceptor derived from a rabbit injected with horse erythrocytes, and rabbit's complement. As human serum contains natural antihorse amboceptor to about the same degree and frequency as antisheep amboceptor, this system is not more reliable than Wassermann's system, and the reagents are less convenient to procure.

Boas advocated a system similar to the Wassermann system in which he used an antigoat amboceptor produced in rabbits. He later abandoned this system.

Browning used an antiox amboceptor, produced in rabbits, and claimed that human serum does not contain a disturbing excess of antiox amboceptor.

Tschernogubow proposed a system in which the natural amboceptor and complement of human serum are utilized against guinea-pig corpuscles. Foix used a system in which the natural amboceptor and com-

[1] Certain proteins, such as peptone, albumoses, nucleoproteins, and certain peptids can often produce, when mixed with unheated human serum, complement fixation closely resembling specific fixation. For this reason active serum should not be used for the test with aqueous or even alcoholic extracts of liver, especially of macerated organs. Pure lipoids free from the substances just mentioned do not give this false fixation with active serum.

plement of human serum are utilized against the corpuscles of the rabbit. The amount of amboceptor that comes into action is indefinite in these two systems.

Kaliski introduced an antisheep hemolytic system in which active patient's serum is used, the native complement of the human serum being reinforced by guinea-pig complement. This system differs from that of Stern in the use of a much smaller proportion of patient's serum (with a consequent reduction of the disturbing effect of the natural antisheep amboceptor and native complement contained in the specimen) and in the employment of guinea-pig complement. This method has now been abandoned in favor of a modified Wassermann technique which makes allowance for the presence of natural antisheep amboceptor.

The systems just described may be considered as of one general order, having in common the use of erythrocytes of animals for which human serum contains an unknown and irregular quantity of natural hemolytic amboceptors. In the systems of Wassermann, Bauer, Boas and Browning guinea-pig complement is used; in those of Hecht, Stern, Tschernogubow and Foix the native complement of patients' serum; in Detre's, that of the rabbit; and in Kaliski's, a mixture of human and guinea-pig complements. In none of these systems can the natural ambocep-

tors be disregarded[1] or quantitatively regulated. In fact, some systems utilize them to produce hemolysis, while others simply disregard their presence and add in every instance a certain amount of immune amboceptor to provide for the occasional absence or deficiency of natural amboceptors.

METHODS IN WHICH HUMAN ERYTHROCYTES

ARE USED

Tschernogubow proposed a system, which he has since abandoned, in which quite a different set of factors is employed, namely, human erythrocytes and antihuman hemolytic amboceptor. He collected the patient's blood (not serum) in saline solution in such dilution that clotting was temporarily prevented. This suspension when fresh contained some complement, erythrocytes, and, if present, the syphilitic antibody. When the antigen (aqueous extract of dried syphilitic liver) was added, the antibody united with it and the combination fixed complement. The antihuman amboceptor was added later, when hemolysis occurred in case the complement had not been fixed. This method had certain disadvantages. The amount of complement in human serum

[1] Certain authors have recommended the removal of the natural amboceptor by means of absorption, that is, the serum is inactivated and mixed with an excess of washed corpuscles, and after thirty minutes' incubation at 37° C. the mixture is centrifuged and the supernatant fluid pipetted off to be used for the test. A considerable amount of labor is involved, especially when hundreds of specimens are handled at a time.

is irregular, and its activity is relatively weak with
regard to antihuman hemolytic amboceptor. The
complement and erythrocytes deteriorate rather rap-
idly, hence no examination can be made of old
specimens. The complement cannot be brought into
contact with antigen alone, and consequently it is
impossible to decide by direct test whether or not
the antigen is inherently anticomplementary. Another
source of error has since been brought to light, that
is, the use of active serum in combination with aque-
ous extracts of liver renders the test nonspecific.
This objection applies to any other system in which
active serum and aqueous or even alcoholic extracts
(unfractionated) of macerated livers are used. Tsch-
ernogubow did not state the source of his antihuman
amboceptor, except that 0.25 c.c. was added to each
tube. This is nearly 125 times the amount of ambo-
ceptor the writer uses in combination with guinea-
pig complement. It is probable that in this way
Tschernogubow overcame, in a measure, the varia-
tions in the amount of human complement present.
Still, the uncertainty in this respect, the impossi-
bility of separating the factors in the system for con-
trol, and, lastly, the necessity of making the test
very soon after the collection of the blood in order
that complement may be active and the erythro-
cytes intact, leave abundant room for improvement
and render the system as outlined unreliable.

After considerable experience with the Wassermann test in its original form, using both the aqueous and the alcoholic extracts as antigen, the writer felt that it would be greatly simplified and made more accurate by eliminating the error due to the irregular presence of natural antisheep amboceptor in human serum. A system in which sheep's corpuscles are used requires fresh washed sheep's corpuscles each time a test is made. Persons far removed from a large abattoir have difficulty in obtaining sheep's blood. Moreover, the serum must be inactivated. By substituting the antihuman hemolytic system for the antisheep it became unnecessary in most instances to inactivate the patient's serum for the test. The writer has recommended the utilization of fresh human erythrocytes obtained from the clot by breaking it up after the serum has been separated for use in the test, or from additional blood drawn at the same time for the purpose. These erythrocytes must be washed with saline solution in order to remove any trace of the serum. When one has become accustomed to judging the concentration of the erythrocyte suspension by sight, it is quite simple to prepare a desired suspension by means of a capillary pipette from the sedimented cells contained in each specimen. In this case there is no necessity for washing the corpuscles, cells and serum coming from the same person. (See p. 89 for strictly quantitative work.)

It has been found by actual titration that the antibody content of a given syphilitic serum is reduced to one-fourth to one-fifth of its original strength after the serum has been inactivated at 56° c. for thirty minutes, hence if the serum is used without inactivation, corresponding results are obtained with very much less serum. Furthermore, the writer has emphasized the importance of using for the test of unheated active serum acetone-insoluble lipoids and not crude alcoholic extract.

The test may be carried out with inactivated sera, this procedure being especially recommended for the examination of specimens which reach the laboratory some time after collection, because inactivation removes the anticomplementary property often present in such sera. Craig, Vedder, and other United States Army officers, who have to deal with a large number of specimens sent from distant army posts, have found that inactivated sera give more satisfactory results than unheated.

It has been found possible to preserve antihuman hemolytic amboceptor in the form of reagent papers, which remain stable for a long time if kept perfectly dry and air-tight and by using an antihuman hemoytic system, the variable antisheep amboceptor of human serum as a disturbing factor is eliminated. The advantage in point of regularity and uniformity, gained by the use of guinea-pig complement, is re-

TABLE IV

Systems	Hemolytic system			Patient's serum	Antigen
	Complement	Amboceptor	Blood-corpuscles		
Wassermann, Neisser and Bruck	Guinea-pig's fresh serum, known definite quantity, 0.1 c.c.	Antisheep amboceptor: that which is present normally in human serum, of variable quantity, and that which is added in form of immune amboceptor (2 units)	Sheep's washed corpuscles, known definite quantity, 1 c.c. of 5 per cent suspension	Inactivated before use. 0.1-0.2 c.c.	Liquid preparation, known adequate quantity. Originally an aqueous extract of syphilitic fetal liver, but Meier later replaced the aqueous extract of syphilitic or normal liver. Later Browning and McKenzie, Sachs, McIntosh and Fildes introduced cholesterin in addition
Bauer*	Do	Antisheep amboceptor. From one source, namely in the serum to be tested. No immune amboceptor added	Do	Do	Do
Hecht*	Utilizes human complement as naturally present in the fresh serum. Old specimens cannot be tested. The activity is rather variable	Do	Do	Tested only in perfectly fresh state without inactivation. Quantity definite but quite large	Alcoholic extract of liver or heart. Non-specific reaction may occur when active serum is used
Gradwohl	Do	Do	0.1 to 0.2 c.c. of washed sheeps' corpuscles, according to the hemolytic index of the serum	Do	Do
Stern*	As in Hecht's system	As in Wassermann's system	Do	As in Hecht's system	As in Hecht's system
Kaliski*	Human complement 0.02 c.c. and guinea-pig's complement 0.03 c.c.	Do	Do 0.1 c.c. 20 per cent	As in the writer's system	As in the writer's system

Systems in which antisheep hemolytic amboceptor is used
GROUP A

* Recorded chiefly because of historic interest.

TABLE IV—Continued

Systems	Hemolytic system			Patient's serum	Antigen
	Complement	Amboceptor	Blood-corpuscles		
GROUP A—Continued					
Detre*	Rabbit's fresh serum, known definite quantity, 0.2 c.c.	Antihorse amboceptor from immunized rabbit, 2 units	Washed horse-corpuscles	Inactivated before use. Known quantity, 0.1–0.2 c.c.	Known adequate quantity in fluid form
Boas*	As in Wassermann system	Antigoat amboceptor, 2½ units	Goat corpuscles 1 c.c., 5 per cent suspension	Do	Alcoholic extract of human heart
Browning*	Do	Anti-ox amboceptor.	Ox corpuscles	Do	Do
Tschernogubow*	Human complement	Anti-guinea-pig amboceptor found in patient's serum	Washed guinea-pig corpuscles	To be tested while perfectly fresh	Aqueous extract of syphilitic liver. Non-specific reaction may occur
Foix*	Do	Antirabbit amboceptor found in patient's serum	Washed rabbit corpuscles	Do	Alcoholic extract of organs
GROUP B — Systems in which guinea-pig precomplement and antihuman hemolytic amboceptor are used					
Noguchi	Guinea-pig's serum, fresh. Definite quantity (2 units) usually 0.1 c.c. of 40 per cent dilution	Antihuman amboceptor from immune rabbits. Liquid or dried preparations used in definite quantity (2 units)	Human corpuscles 0.1 c.c. of 10 per cent suspension	Either fresh serum, 0.02 c.c., or inactivated, 0.1 c.c., may be used	Adequate quantity in liquid form. With inactivated serum activated serum or alcoholic extract or pure acetone-insoluble lipoids can be used. With active serum only the pure lipoids must be used, in order to avoid non-specific reaction
Craig	Do	Do	Do	Inactivated serum	Alcoholic extract of liver of syphilitic fetus

* Recorded chiefly because of historic interest.

TABLE IV—Continued

| Systems | Hemolytic system | | Blood-corpuscles | Patient's serum | Antigen |
	Complement	Amboceptor			
Tschernogu- bow*	Human complement as present in the patient's blood. Variable	Antihuman amboceptor. Large quantity required	Human corpuscles, not washed, and containing fibrin ferment and fibrinogen	Tested only while perfectly fresh. Quantity not adjustable	Aqueous extract of dried syphilitic liver. Non-specific reaction may occur
v. Dungern*	Human complement contained in 0.1 c.c. of defibrinated blood and guinea-pig complement dried on paper possessing definite activity	Antihuman amboceptor from immunized goat	Human corpuscles contained in 0.1 c.c. of defibrinated blood, unwashed	Tested only while perfectly fresh. The amount of serum is that contained in 0.1 c.c. of defibrinated blood	Alcoholic extract of guinea-pig heart. Occasional non-specific reaction may occur
Emery	Human complement contained in 0.1 c.c. of defibrinated patient's blood	Antihuman amboceptor (rabbit)	Human corpuscles contained in 0.1 c.c. of defibrinated blood	Tested only while fresh	Alcoholic extract of organs plus cholesterin. Non-specific reaction may occur
Butler and Landon	Human complement, definite quantity from a nonsyphilitic person	Antihuman amboceptor (rabbit)	Washed human corpuscles only	Inactivated serum only	Acetone-insoluble lipoids only
Thompson	Human complement, usually 0.2 c.c., to be titrated	Antihuman amboceptor (rabbit)	0.2 c.c. of a 2 per cent suspension of human corpuscles	Tested while fresh	Acetone-insoluble lipoids only
Noguchi	Human complement contained in 0.2 c.c., supplemented when occasionally insufficient by another specimen of active human complement known to give negative reaction	Antihuman amboceptor (rabbit)	Human corpuscles washed if from other specimens, not necessarily washed if from the same specimen of blood as the serum. 0.1 c.c. of 10 per cent suspension	Fresh specimens only can be tested	Acetone-insoluble lipoids only

GROUP C
Systems in which human complement and antihuman hemolytic amboceptor are used

* Recorded chiefly because of historic interest.

tained without any sacrifice in accuracy. The details of the method, with directions for preparing the reagents for use and the detailed directions for carrying out the test and interpreting the results, have been brought together in another chapter of this book. The matter presented with regard to the different forms of the complement-fixation test is summarized in Table IV, on pp. 49, 50 and 51.

Von Dungern once introduced a system somewhat similar to that of the writer. Like Tschernogubow, however, he used the patient's blood in the active state only, for the reason that he utilized the erythrocytes of the same specimen, and inactivation was, therefore, not applicable. The blood was defibrinated before use, but the native complement contained in it was not used as the complement constituting the hemolytic system. He advised the use of guinea-pig's serum in dried paper form. The amount of the defibrinated blood used for the test was 0.1 c.c., a rather large quantity, and the presence of the native complement can hardly be left out of consideration. Moreover, crude alcoholic extract (without fractionation) is used as antigen, hence the possibility of obtaining occasional non-specific proteotropic fixation with active human sera. The amboceptor used by von Dungern was derived from the immunized goat.

CHAPTER VII

Inactivation of the Serum in Relation to the Syphilis Reaction

ACCORDING to the method used in the systems of Wassermann, Detre, Bauer, Boas, and Browning the serum is previously heated to 56° c. for half an hour, in order to destroy all the native complement present in it. On the other hand, several systems have been mentioned in which the serum is used in the fresh state in order to utilize the native complement. The writer's original antihuman system, unlike any of these two sets of systems, enables one to use either fresh or inactivated serum, the only difference being that when the serum has been inactivated a quantity about four or five times as large must be employed.

Wassermann, Bauer, and others inactivate the serum simply to destroy the native complement, which varies in different specimens of serum, and to substitute this unknown content by a uniform amount of guinea-pig complement of known activity. Hecht and Stern, however, found that when the test is made with fresh serum, so as to employ the native complement, the reaction is more sensitive than when the inactivated serum is used. On what does this greater delicacy of reaction of the fresh serum de-

pend? The writer made a careful analysis of the difference existing between fresh and inactivated serums and arrived at the following conclusions.

The first question was: Is the so-called syphilitic antibody affected in the process of inactivation? It had previously been found that this antibody is completely destroyed at temperatures between 72° and 80° c. in about twenty minutes, that is, we knew with fair accuracy the limit of temperature
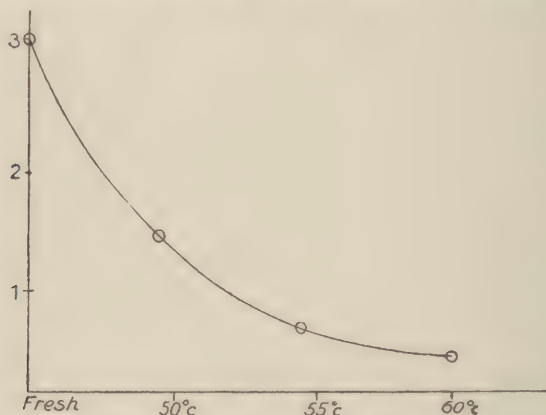
CURVE 1. Rate of reduction of syphilitic antibody in a serum heated to different temperatures for twenty minutes (in a water-bath).

at which the total destruction of the antibody is effected, but we did not know much about the rate of destruction which takes place at lower temperatures. The writer, therefore, undertook a series of experiments to determine the rate of destruction of the so-called syphilis antibodies through heat by subjecting several specimens of positive syphilitic

serum to different temperatures for varying lengths
of time. In the accompanying curve is shown the
effect of heating maintained for twenty minutes upon
the antibody content of a specimen of serum from
a case of untreated secondary syphilis. The deter-
mination is of course made by fixation tests. It was
found that syphilitic antibody is greatly reduced
even at 45°. At 50° c. it is reduced to about one-half,
at 55° c. to about one-fourth, etc. In another series
of experiments tests were made to determine the
rate of destruction of the antibody at the temperature
of 55° c., at five, ten, twenty, thirty, and sixty
minute periods. The results were rather unexpected,
since the rate of destruction is greatest during the first
five minutes, during which time the antibody strength
is reduced to about one-third of the original. After
thirty minutes it has been reduced one-fourth to
one-fifth, and at the end of one hour to about one-
tenth of the original, as can be seen by reference to
Curve 2. In studying the serum from a case of lep-
rosy, it was found that the diminution of the antibody
strength went on in precisely the same way as in the
specimens obtained from syphilis (Curve 3).

From the foregoing experiments it follows that in
employing fresh unheated serum one is using about
4 to 5 times as much so-called syphilis antibody as
when the same quantity of inactivated serum is used.
Another important fact with regard to inactivation

of the serum is the claim made by some investigators that the fresh serum of nonsyphilitic cases—as, for example, cases of carcinoma—may give a positive reaction, that this reaction disappears when such a
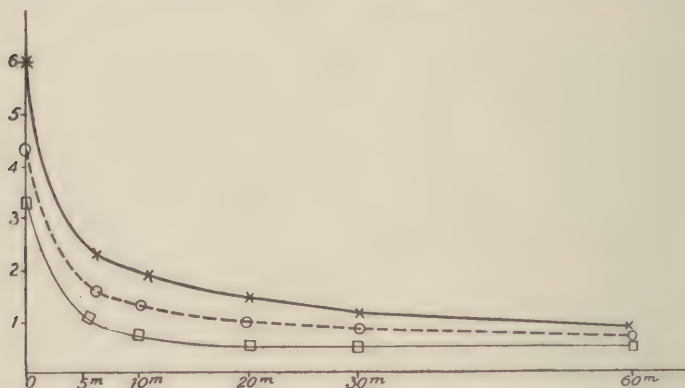
CURVE 2.   Rate of reduction of syphilitic antibody in three different samples of syphilitic sera heated to 55° C. for varying lengths of time (in a water-bath).

serum has been previously heated to 56° c. for thirty minutes, and that therefore the two groups of positive reaction, specific and nonspecific, can be distinguished by the employment of inactivated serum.

The writer found that this assertion holds true only when the preparations serving as antigen contain certain proteins present in the alcoholic extract of heart or liver such as are ordinarily employed by some laboratories. This peculiar fixation phenomenon is not confined to the above instance alone, but occurs whenever the antigens consist of protein matter. For example, while studying the Bordet-

Gengou fixation phenomena in general, the writer
met with the unexpected fact that in the majority
of active human sera, irrespective of sources, there
exists a constituent which fixes complement when
mixed with certain proteins such as nucleoproteins,
peptone, albumoses, and various other autolytic
cleavage products of proteins. This phenomenon
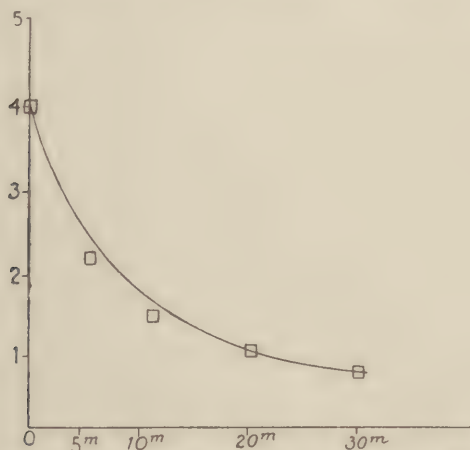
CURVE 3.   Reduction of antibody due to heating at 55° C. serum from a
case of leprosy.

was designated proteotropic fixation in contradis-
tinction to the lipotropic fixation due to the action
of certain lipoids upon the syphilitic serum. The
proteotropic fixation is of course non-specific,
but is difficult to differentiate from the actual
specific Bordet-Gengou as well as the Wasser-
mann reactions. Fortunately this proteotropic reac-
tion does not occur when the serum is previously

heated to 56° c. for twenty minutes, while the lipo-
tropic substance of syphilitic serum is reduced but
not destroyed. Considering the Wassermann reac-
tion in the light of this finding it becomes at once
clear that only inactivated sera should be used for
the test when aqueous or alcoholic extracts of organs
are employed as antigens, because these preparations
contain various proteins and are likely to give a non-
specific proteotropic fixation with active sera. There
is no danger, however, in using active sera when pure
lipoidal substances are used as antigen. The experi-
ences of many investigators demonstrate the correct-
ness of this point, and for this reason the writer
emphasizes the importance of using acetone-insolu-
ble tissue lipoids for antigen. The introduction of
pure lipoidal antigen rendered it for the first time
possible to employ an active human serum for the
diagnosis of syphilis. It is obviously unwise to em-
ploy preparations of antigen containing various
proteins in any method in which active sera are prin-
cipally used. The results obtained by using active
human serum and the lipoids just referred to are
comparable with those obtained by inactivated
serum and any other suitable syphilitic antigens and
are entirely specific.[1]

[1] It is an erroneous conception that only active serum is used in the
writer's system. On the contrary, inactivated serum can be used as well,
the matter being one of personal choice, provided that the rules prescribed
for active and inactive sera are observed.

CHAPTER VIII

A SYSTEM OF SERUM DIAGNOSIS OF SYPHILIS RECOMMENDED BY THE AUTHOR

IN the following pages it is my purpose to present as briefly as is consistent with the necessary detail the method of making a diagnosis of syphilis by serum reaction which has been developed in my laboratory. The presentation will, it is hoped, be of interest and service to two distinct sets of investigators: the practising physician who is so trained that he can make his own clinical laboratory tests, and the laboratory worker who is concerned either in making laboratory diagnostic tests for others or in supplying laboratory reagents in convenient and stable form for others to use.

It was my desire to improve and simplify the original method to such an extent that it might be carried out by those who, although not equipped to prepare the reagents themselves, can nevertheless carry out the test reliably. The original method of Wassermann was difficult to carry out chiefly because the reagents were rather unstable and were associated with so many undesirable incidental effects that they must be used soon after being prepared. This required that the performer know how to prepare the reagents and to make the test independently. These difficulties were inherent in most of the

modifications already described. On the other hand, if we could develop the method in such a manner that the preparation of the most important and difficult reagents would be done by competent laboratory workers in a regular biological laboratory and distributed among those who understand how to use them, no abuse of the test could occur. The writer believes that the reagents (amboceptor and antigen) recommended in his system remain unchanged for a very long period; nevertheless the performer of the test should know how to determine the reliability of the reagents he secures from others. The presentation is accordingly made in two distinct sections:

A description of the method of preparing the reagents, standardizing them, and preserving them in stable form.

A description of the technique.

METHODS OF PREPARATION AND TITRATION OF REAGENTS

COMPLEMENT

Guinea-pig's serum is used. Large animals are selected and are bled by cutting the carotid artery, allowing the blood to flow into a large Petri dish. The dish is covered and left at room temperature for a few hours for the clot to form and the serum to separate. If desirable for economic reasons, a number of guinea-pigs may be kept in the laboratory, and

2–3 c.c. of blood may be drawn by heart puncture from each of several animals and the pooled serum used as complement. The animals so bled may be bled again within three or four weeks.

The separation of the serum may be completed in the refrigerator. Within eighteen hours all the serum has separated from the clot and should then be poured into a sterile test-tube and thereafter when not in use kept in the refrigerator. After the serum is forty-eight to seventy-two hours old the activity of complement is rapidly lost, even at refrigerator temperature. Whenever possible the use of unmodified serum is advisable, but several methods of preservation now in use will be described.

0.1 c.c. of 40 per cent dilution is used.

Rhamy's Method of Preserving Complement. Guinea-pig serum may be preserved by mixing 6 parts of 10 per cent sodium acetate solution with 4 parts of fresh complement. The mixture remains active for at least two weeks when kept at a low temperature (below 10° C.); it is understood, however, that its activity requires careful titration before use.

Use of Sodium Chloride as Preservative. Sodium chloride has been added to undiluted guinea-pig complement in a concentration of 17 per cent, 0.17 gm. of pure crystal sodium chloride being dissolved in each c.c. of serum and the mixture kept at 4–6° C. At the time of use, each c.c. of serum is diluted

with 19 c.c. of distilled water, yielding a 1:20 dilution of complement in isotonic saline solution. The same procedure has been followed with one-half the amount of sodium chloride, in which case 9 c.c. of water is added afterwards, making the dilution 1:10 (Kolmer).

Thompson recommended a 1:1 dilution preserved with 8.1 per cent sodium chloride, Austin a 40 per cent dilution of complement with 25 per cent sodium chloride, and Neill 1 c.c. complement with 0.1 c.c. of saturated sodium chloride solution. Ruediger recommends the preservation of complement with glycerol, 3 parts serum to 1 part of sterilized glycerol.

The writer has experimented with Rhamy's sodium acetate method and with various sodium chloride methods, and has found that at a temperature of about 6° c. the complement remains active for at least one week. The authors of these methods have found the complement to be undiminished in activity even after a period of two to three weeks.

The use of dried complement, as earlier recommended by the writer, has been abandoned because of the considerable reduction in activity by the process of drying, marked deterioration occurring as soon as one week.

AMBOCEPTOR

Antihuman hemolytic amboceptor is used. It is made by immunizing rabbits against human blood-

corpuscles. Large rabbits are given five intraperi-
toneal or intravenous injections, at four- or five-day
intervals, of increasing amounts of washed[1] human
blood-corpuscles. Nine or ten days after the last in-
jection, the rabbits are bled from the carotid artery.[2]

<div align="center">

TABLE V

SCHEDULE FOR IMMUNIZATION

</div>

*Injections at four- or five-day intervals.   Bleeding nine or ten days after the
last injection*

        1st injection,   5 c.c. intraperitoneally or 3 c.c. intravenously
        2d injection,   8 c.c. intraperitoneally or 3 c.c. intravenously
        3d injection, 12 c.c. intraperitoneally or 4 c.c. intravenously
        4th injection, 15 c.c. intraperitoneally or 4 c.c. intravenously
        5th injection, 20 c.c. intraperitoneally or 4 c.c. intravenously

After the blood is collected the blood-tube is
placed at room temperature for several hours, during
which period the clot gradually contracts and sepa-
rates from the wall of the tube, allowing a clear serum
to exude into the space between the clot and the wall
of the tube. If the clot remains uncontracted within

[1] The corpuscles must be washed at least three times with a large amount
of saline solution. If this is not done the immune serum may contain
precipitin for human serum, which will interfere with the fixation reaction.

[2] Vedder has suggested a method of producing antihuman hemolysin
by immunizing rabbits with the stroma of the red corpuscles. The packed
corpuscles are diluted with equal parts of 0.85 per cent NaCl, and illumin-
ating gas is allowed to bubble through the mixture for fifteen or twenty
minutes, then the salt solution is centrifuged off and enough distilled water
added to produce hemolysis. After an hour's standing the stroma (about
33 per cent of the corpuscles) are separated by centrifugation at high speed
and diluted with salt solution to the original volume of the packed cor-
puscles. A first injection of 1 c.c. of this suspension is followed at five-day
intervals by three more injections of 2 c.c.

four or five hours, it is carefully separated from the wall by inserting a sterile stiff platinum needle or glass rod, and allowed to stand at room temperature for several hours longer to promote the contraction of the clot. The blood is then placed in a refrigerator for twenty-four hours. The clear serum is collected by decantation or by centrifugation. If contraction of the clot is not complete, the tube may be left for another day and more serum collected, the portions of the serum collected in this manner being mixed together. The corpuscles may be sedimented by further standing or by centrifugation.

Method of Titrating Amboceptor. The amboceptor may be used in fluid form or may be impregnated on paper, but in either case had best be titrated at time of use.[1] The principles of the titration have been fully discussed pp. 8–10, 31–35). The procedure is illustrated by Table VI. A series of successive tenfold dilutions of the serum is made as follows: Mix 1 c.c. of the serum with 9 c.c. of salt solution; mix 1 c.c. of this 1:10 dilution with 9 c.c. of salt solution (1:100 dilution), repeating the process to get a 1:1000 dilution. These three dilutions will enable one to measure out any quantity desired for titration. From personal experience the writer has found

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[1] Different rabbits react differently to amboceptor production and it is not rare to get a weak serum after long immunization, or a powerful serum after only four injections.

the following scheme of graduated doses suitable (Table vi).

TABLE VI

SAMPLE TITRATION OF AMBOCEPTOR OF UNKNOWN STRENGTH

Tube no:	Absolute quantity c.c.	Quantity of dilution, c.c.	Saline needed to make total volume I c.c.	40% complement	10% corpuscle suspension	Result after 30 minutes at 37° C. in water-bath
I	0.05 =	0.5 of 1 : 10	0.5	0.1	0.1	Complete hemolysis
2	0.03	0.3	0.7	0.1	0.1	Complete hemolysis
3	0.02	0.2	0.8	0.1	0.1	Complete hemolysis
4	0.015	0.15	0.85	0.1	0.1	Complete hemolysis
5	0.01	1.0 of 1 : 100		0.1	0.1	Complete hemolysis
6	0.007	0.7	0.3	0.1	0.1	Complete hemolysis
7	0.005	0.5	0.5	0.1	0.1	Complete hemolysis
8	0.004	0.4	0.6	0.1	0.1	Complete hemolysis
9	0.003	0.3	0.7	0.1	0.1	Complete hemolysis
10	0.002	0.2	0.8	0.1	0.1	Complete hemolysis
11	0.0015	0.15	0.85	0.1	0.1	Complete hemolysis
12	0.001	1.0 of 1 : 1000		0.1	0.1	Complete hemolysis (for test use this dose = 2 units)
13	0.0007	0.7	0.3	0.1	0.1	Complete hemolysis
14	0.0005	0.5	0.5	0.1	0.1	Complete hemolysis (this is the titre, or one unit)
15	0.0004	0.4	0.6	0.1	0.1	Partial hemolysis
16	0.0003	0.3	0.7	0.1	0.1	Slight hemolysis
17	Saline control		1.0	0.1	0.1	No hemolysis

The tubes must be filled up to a uniform volume of

5

1 c.c. each by adding salt solution, as indicated in the table. A good preparation will have a value of 1 unit in something less than 0.001 c.c. of serum, that is, 0.001 c.c. of the amboceptor serum or less will cause complete hemolysis of 0.1 c.c. of a 10 per cent suspension of human erythrocytes when combined with 0.04 c.c. of guinea-pig's fresh serum (0.1 c.c. of a 40 per cent dilution). Should complete hemolysis occur in the dose of 0.0005 c.c. (14th tube in the schedule), this quantity represents the titre. The titre may, however, be as low as 0.005 c.c., or even 0.01 c.c. Subsequent titrations of the same serum may be made by using three doses above and one or two below the titre already found. Two units are used in the test.

Method of Impregnating Paper with Amboceptor. When the amboceptor preparation is of the required strength, as will usually be the case if the method of immunization outlined is followed, we may proceed to the preparation of the paper. This is a simple matter, as the amboceptor is stable. As the slip of paper will contain only about 0.001 c.c. of serum a thin filter paper is satisfactory, such as Schleich and Schull's paper No. 597. It is cut into squares of about 10 × 10 cm. which are placed in a dish containing sufficient serum to saturate them. The excess is drained off by touching the edge of the sheet to the edge of the dish on withdrawal. One should

avoid too great an excess. The paper is dried at room temperature by placing each square separately upon a clean sheet of non-absorbent (unbleached) muslin. Several hours' drying usually suffices. When thoroughly dry the sheets are cut into strips of convenient width, 5 mm. for example, and then standardized.

Titration of the paper is made as follows: To a series of tubes, each containing 1 c.c. of the 1 per cent erythrocyte suspension and 0.1 c.c. of a 40 per cent dilution of complement, measured increasing lengths of the amboceptor strip are added, *e.g.*, 1 mm. for the 1st tube, 2 mm. for the 2nd, 3 mm. for the 3rd, etc., and the tubes are incubated for 30 minutes in the water-bath or for 1 hour in an air thermostat at 37°. The shortest strip which causes complete hemolysis in this time contains 1 amboceptor unit. The strips are then marked into sections of *twice* this length and cut off at the time of doing the test. Each section will then contain two units. The papers should be kept dry and sealed.

<div align="center">ANTIGEN</div>

It has now been demonstrated that alcoholic extracts of certain tissues contain variable quantities of "antigen" for syphilis. There is more in heart, liver, or kidney than in nervous tissues, so far as has been determined. The liver of a congenitally syphi-

litic fetus was once considered richest in these antigenic lipoids, but later investigations showed that it is not superior to other tissues in this respect. It has been found that not only selected samples of tissue phosphatids, but also several acetone-soluble fractions of tissue lipoids, can act as antigen. Whether the extract is derived from syphilitic organs or from non-syphilitic tissues makes but little difference; it is possible to obtain good preparations from animal tissues just as frequently as from human organs. In all cases one must determine the antigenic quality of the extract before using it as antigen.

Two essentially different preparations are used today: (1) Plain alcoholic extract of heart or liver of man, ox, or horse, sometimes of the guinea-pig; (2) The acetone-insoluble fraction of tissue lipoids introduced by the writer in 1910. The difference between these two preparations consists in the presence in the former of various fatty acids or their salts, cholesterin, neutral fats, and certain protein substances, in addition to the acetone-insoluble lipoids which alone make up the latter. The acetone-insoluble antigen is derived from the plain alcoholic extract, all other constituents except the phosphatids having been gradually eliminated by successive fractionation. From the serological standpoint the following facts deserve careful consideration before the selection of an antigen preparation:

(1) All the antigenic substances contained in a plain alcoholic tissue extract are contained *in toto* in that fraction which is insoluble in acetone. (2) The fatty acids or their salts (soaps) which are present in a plain alcoholic extract are hemolytic and also interfere with the activity of complement. (3) The alcohol-soluble and ether-insoluble fraction of a plain alcoholic extract, often twice as great in quantity as all other substances contained, has no hemolytic action, but it contains some substances which interfere with the activity of complement when mixed with some specimens of unheated human serum.

It is evident, then, that in a method in which unheated human serum is being used for the test a plain alcoholic extract is to be avoided for the reason that it may produce a false positive fixation with a negative specimen. In tests of unheated serum, as has been emphasized previously, a purified acetone-insoluble lipoidal fraction should always be employed. Experience has shown that a suitable acetone-insoluble antigen accomplishes all that it is possible to accomplish with a plain unfractionated alcoholic extract in combination with inactivated serum. In other words, a plain alcoholic extract may be used only with inactivated serum, whereas the acetone-insoluble fraction is applicable both to inactivated and to unheated serum. The following diagram will perhaps illustrate this point more clearly:

Beef (or human) heart

Alcoholic extraction at 37° C. for several days

Filtrate (this is the usual alcoholic extract antigen)

Evaporated to dryness, resulting in a sticky brownish residue

Treated with ether to extract all soluble matter

Ether-insoluble residue sticky, gummy, grayish brown substance and salts. This causes a non-specific fixation when mixed with unheated serum.

Ether soluble fraction

Concentrated by evaporation

Treated with acetone to separate into

Acetone-soluble fraction.

This contains fat, cholesterin and other acetone-soluble fatty substances which have a hemolytic or anticomplementary action.

Acetone-insoluble fraction.

This represents a highly antigenic, stable substance, usually devoid of hemolytic or anti-complementary properties. This fraction, when found by titration to fulfill the requirements already stated, is that recommended by the writer to be used as antigen.

PREPARATION OF ACETONE-INSOLUBLE ANTIGEN

Original Method (1910). The writer's original method of preparing the acetone-insoluble extract is as follows:

Extract a mashed paste of heart of ox or calf with 10 parts of absolute alcohol at 37° c. for several

days. Filter through paper and collect the filtrate. The latter is evaporated with the aid of an electric fan, the residue is taken up with ether, and the turbid ethereal solution is allowed to stand over night in a cool place; the receptacle must be covered during this period in order to prevent evaporation of ether. The next morning it will be found that the turbidity has entirely cleared up by gravitation of the insoluble particles to the bottom. The clear ethereal portion is then carefully decanted into another clean beaker and condensed into a small quantity by evaporating the ether. The concentrated ethereal solution is now mixed with about ten volumes of pure acetone.

A precipitate forms, which is allowed to settle to the bottom of the vessel, and the supernatant fluid is decanted off, leaving a light brownish precipitate which gradually becomes sticky on exposure to the air. This acetone-insoluble portion of the tissue extract contains antigenic lipoids, and its quality and strength must be determined. If we start with 300 gm. of beef heart it is possible to obtain about 7 gm. of this fraction. If the preparation proves to be suitable as antigen this quantity will supply enough antigen for more than 20,000 tests.

Stock Solution. 0.2 gm. of the acetone-insoluble fraction is dissolved in about 1 c.c. of ether in a test-tube. The ethereal solution is then mixed with 9 c.c.

of methyl alcohol in which the greater part of the substances goes into solution. The alcoholic solution, which contains 2 per cent of the lipoids, remains unaltered for a long time and can be kept as stock from which the emulsion for immediate use may be prepared at any time. When a large quantity of stock solution is made it is advisable to keep the greater portion set aside in a tightly sealed brown bottle. Oxidation reduces the antigenic strength of this fraction. Samples thus preserved have remained unaltered for many years at ordinary laboratory temperature.

Second Method (1921). *Stock Solution.* Beef heart is ground in a sausage machine, and rapidly dried by an electric fan. Three hundred grams of the dried substance are extracted with 1 liter of acetone for five days at room temperature, with daily shakings. The acetone is then discarded, and the mass of solids freed from acetone by evaporation is extracted with 1 liter of absolute alcohol for five days at room temperature. The golden yellow alcoholic extract, which contains acetone-insoluble tissue lipoids has been found to be an excellent antigen (see p. 225).

Emulsion of Antigen. This should be prepared by adding, drop by drop, 9 c.c. of 0.9 per cent saline solution to 1 c.c. of the alcoholic stock solution, the mixture being shaken thoroughly each time a drop is added.

In the case of antigen prepared by the writer's original method (1910), this procedure will result in an opaque, whitish emulsion; if the mixing is done rapidly without shaking, a clear opalescent emulsion results which gives a weaker reaction than the opaque emulsion and is not recommended. In the case of the writer's second (dry) method, or Bordet's method (described below) the emulsion with saline is never very turbid, and is usually opalescent, yet has equally powerful antigenic property. (See footnote, p. 225.)

Bordet's Method. Bordet accepts the writer's view that the acetone-insoluble tissue lipoids are best suited for the serum reaction in syphilis. He has recommended another procedure, which is not only economical but also yields a highly satisfactory "antigen." 100 grams of chopped calf heart are shaken with 125 c.c. of 95 per cent alcohol and the mixture allowed to stand for several days at room temperature. It is then filtered and the tissue spread out in a large crystallizing dish and dried in the incubator at 37° C. for twenty-four hours. 200 c.c. of acetone is then added to the dried tissue and allowed to remain for a week at room temperature. The acetone is replaced by an equal quantity of fresh acetone, which is allowed to remain twenty-four hours. The filtered tissue is freed from acetone by drying for several hours in the incubator and

then extracted for eight to ten days in 95 per cent alcohol at room temperature. The golden yellow filtrate is the antigen. At the time of using 0.5 c.c. of the alcoholic extract is evaporated on a watch glass, and to the yellowish residue is gradually added 2 c.c. of distilled water. The liquid is transferred to a tube and 18 c.c. of isotonic NaCl added while shaking. The resulting fluid is slightly opalescent.

Neymann and Gager's Method. Neymann recommended a somewhat different process for extracting the acetone-insoluble fraction of lipoids. The method consists in first desiccating heart muscle and then subjecting the dried powder to extraction with ether and absolute alcohol. The last (alcoholic) extract is then dried and fractionated with acetone. The acetone-insoluble portion of the lipoids is used for the Wassermann reaction.

SELECTION AND TITRATION OF ANTIGEN

Before entering into the technical details of titration of the antigenic lipoids, certain properties of this fraction in general may be discussed. Usually the acetone-insoluble fraction of tissue lipoids as prepared by the method just described has no *hemolytic action* upon human, or any other animal, erythrocytes, but one occasionally encounters a preparation which

causes hemolysis when used in a large amount. The presence of such a property in the preparation renders it unsuitable for antigen, hence it is necessary to test every sample of extract for hemolytic property. Another property rendering the extract unsuitable is *anticomplementary action*, that is, the property to diminish or destroy the activity of complement. In a very large amount this anticomplementary effect may be present in nearly 50 per cent of different samples; hence, every specimen must be examined for its anticomplementary activity. If it exceeds a certain limit, to be specified later, the specimen is not to be employed as antigen.

After eliminating those preparations which are either hemolytic or anticomplementary or both by properly arranged series of determination we come to the question of *antigenic property*, by which is meant the power to fix complement in the presence of syphilitic serum. This most important property of the extract is very variable in different samples. According to a systematic analysis of nearly 100 different specimens, carried out by the writer and Bronfenbrenner, about 50 per cent were found to be serviceable and only about 5 per cent were altogether devoid of antigenic property. The latter were all from fatty livers. The other 45 per cent were not suitable because of pronounced anticomplementary and weak antigenic properties.

A titration of antigen therefore includes the determinations of (a) hemolytic, (b) anticomplementary, and (c) antigenic activity and can be made simultaneously in a properly arranged series of experiments. The following is the method which the writer recommends.

CHART I

Tube 1 (Test for hemolytic property)
Antigen emulsion (1:10 dilution)...................... 0.4 c.c.
Salt solution... 0.6 c.c.
Corpuscle suspension (10 per cent)...................... 0.1 c.c.
Incubation at 37° C. for one hour in an air thermostat or for thirty minutes in a water-bath.

Tube 2 (Test for anticomplementary property)
Antigen emulsion (1:10 dilution) .... 0.4 c.c.
Salt solution.. 0.6 c.c.
Complement (40 per cent).............................. 0.1 c.c.
Amboceptor .... 2 units
Incubation at 37° C. for one hour or for thirty minutes in a water-bath.
Corpuscle suspension (10 per cent) 0.1 c.c.
Incubation at 37° C. for one hour in an air thermostat or thirty minutes in a water-bath.

Tube 3 (Test for antigenic property)
Antigen emulsion (1:100 dilution) .... 0.2 c.c.
Salt solution.. 0.8 c.c.
Syphilitic serum............. 0.1 c.c. inactivated or 0.02 c.c. active
Complement (40 per cent) .... 0.1 c.c.
Amboceptor... 2 units
Incubation at 37° C. for one hour or for thirty minutes in a water-bath.
Corpuscle suspension (10 per cent) 0.1 c.c.
Incubation at 37° C. for one hour in an air thermostat or for thirty minutes in a water-bath.

CHART II

VARIETIES OF ACETONE-INSOLUBLE LIPOIDAL PREPARATIONS WHICH MAY BE ENCOUNTERED WHILE TESTING FOR A SATISFACTORY SAMPLE OF ANTIGEN

	Hemolytic property (0.4 c.c. emulsion)	Anticomplementary property (0.4 c.c. emulsion)	Antigenic property (0.02 c.c. emulsion)	Remarks
1st group (satisfactory)..........	−		+	Suitable
2nd group (single defect).........	−	−	<+	Unsuitable because of weakness of antigenic property
3rd group (single defect).........	−	−	−	Unsuitable because of lack of antigenic property
4th group (single defect).........	−	+	+	Unsuitable because of anticomplementary property
5th group (single defect).........	+	−	+	Unsuitable because of hemolytic property
6th group (double defects)........	+	+	+	Unsuitable because of hemolytic and anticomplementary property
7th group (double defects)........	+	−	−	Unsuitable because of hemolytic property and lack of antigenic property
8th group (triple defects)........	+	+	−	Unsuitable because of hemolytic and anticomplementary properties and of absence of antigenic property

− = absent; + = present; <+ = weakly present.

Technique. It has been found by long experience and many experiments that 0.4 c.c. of the 1:10 emulsion in saline solution is sufficient for testing the hemolytic and anticomplementary properties, and 0.02 c.c. for testing antigenic power. In measuring such a small quantity as 0.02 c.c. of emulsion it is of course necessary first to prepare a tenfold dilution, with salt solution, of the usual emulsion (1:10 dilution) and 0.2 c.c. of the dilution will correspond to 0.02 c.c. of the regular emulsion. The procedure of determination is shown in Chart 1.

These three determinations can be made at the same time in a series of experiments; and it is not necessary to wait the result of one determination before commencing the others. Any one of the possibilities shown in Chart 11 may be obtained.

The Quantity of Antigen for Fixation Test. It is essential (pp. 79–80) to employ more than four antigen units. To fulfil this requirement the writer recommends the use of 0.1 c.c. of a 1:10 saline suspension of the acetone-insoluble tissue lipoids whose suitability as antigen has previously been determined in the manner already described. In this quantity of the emulsion there will be at least five antigen units (0.1 c.c. ÷ 0.02 c.c. = 5).

Examination of the quantitative relationship between the amount of emulsion employed for testing the hemolytic and anticomplementary properties

and that used for testing antigenic property will show that it now stands 20:1. This renders the fixation test absolutely free from possible interference by the anti-complementary or hemolytic activity of the antigenic preparation alone. It may not be amiss to note here Wassermann's requirements for the selection of a preparation of extract for the test. His rule is first to determine the minimal quantity of extract which inhibits hemolysis completely and then to find out whether or not half of this anticomplementary dose gives complete fixation with a syphilitic serum. If it does, the preparation is considered suitable. This quantity of extract may contain one antigen unit or several, and one goes on with the fixation test without knowing how many antigen units he is employing. If the results obtained with one unit of antigen were the same as those obtained with several there would be no chance of variation in the results, but, in reality, the reactions differ considerably according as one or several antigen units are used. This is especially so with a weak syphilitic serum. In the writer's opinion it is absolutely necessary to use several antigen units in order to get uniform results with any specimen, because the weakest reaction escapes detection unless one employs more than four antigen units. It is understood, however, that an oversensitive reaction must not be allowed to occur from the use of too many antigen units. The use of

a suitable antigen—prepared by the method here described—has never in the writer's experience caused undue sensitiveness. The same principle does not apply to crude alcoholic extracts, either plain or cholesterinized, because they always contain a much larger quantity of the anticomplementary substances.

THE USE OF CHOLESTERIN AS A REINFORCEMENT FOR SYPHILIS ANTIGEN

Browning and McKenzie found that when a certain quantity of cholesterin is added to an alcoholic solution of lecithin the antigenic strength of the latter is greatly intensified. In fact, by the addition of cholesterin in a ratio of about 0.4 per cent, which is near saturation in alcohol at 37° C., almost any preparation of lecithin may become a serviceable antigen. Sachs, and McIntosh and Fildes found that a plain tissue extract, human heart extract, for example, gives a more reliable result than a pure lecithin. McIntosh and Fildes recommended the reinforcement of the ordinary crude extract by means of cholesterin in order to obtain a greater number of positives among syphilitics, especially in those suffering from latent or slowly progressing nervous affections (tabes dorsalis), or those under antisyphilitic treatment. Swift and others have confirmed and extended these observations. According to the majority of investigators, the cholesterinized antigen,

through its intensified reaction, gives valuable information to one following the effect of treatment of syphilis, but it is not reliable in a diagnosis of an unknown case because it may give a false positive reaction. It has therefore become the custom in certain laboratories to carry on two sets of tests with each serum, one with ordinary antigen for diagnosis, and the other with cholesterinized antigen for prognosis.

Cholesterinized Acetone-insoluble Lipoidal Antigen. The writer has pointed out elsewhere that a positive reaction rarely escapes detection when a suitable sample of acetone-insoluble antigen is used. The results stand between those obtained with a cholesterinized alcoholic extract and those with the ordinary crude alcoholic extract. Nevertheless, by adding cholesterin to the acetone-insoluble antigen in a ratio of 0.4 gm. to 100 c.c. of methyl alcohol stock solution of the latter, the reaction is in some instances intensified.

It is important, however, to take into consideration the manner by which the emulsion of the antigen is prepared, that is, whether saline solution is poured into a given quantity of the methyl alcoholic solution of the acetone-insoluble lipoids rapidly without shaking or is added drop by drop, with a thorough shaking for each drop, because the antigenic value of the same sample of acetone-insoluble lipoids may

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differ greatly according to the manner of preparation of the saline emulsion. By the former method a faintly bluish white opalescent emulsion is obtained, while by mixing saline drop by drop with constant shaking we usually get a whitish opaque emulsion. (For exceptions, however, see p. 73.) These two emulsions, containing exactly the same quantity of lipoids and saline solution, are quite different in their physical composition and behave differently with respect to the Wassermann reaction. Actual titration of them for their antigenic power shows that the reaction with the milky opaque emulsion (made by thorough shaking) is about four times as strong as that obtained with the opalescent emulsion (rapid mixing) for the same specimen of syphilitic serum. In other words, a serum which just gives complete fixation with the opaque emulsion reacts only weakly or even negatively to the opalescent emulsion. In order to obtain complete fixation with the latter, it is necessary to employ about four times as much serum.

Now, reverting to the reinforcement by means of cholesterin, it has been found that if cholesterin has been added to the methyl alcoholic stock solution of acetone-insoluble lipoids dispersion can be brought about on mixing with saline without shaking, as shown by the semitransparent whiteness of the un- shaken emulsion. Such an emulsion intensifies the

reaction nearly four to five times as compared with that obtained by the use of an opalescent emulsion without cholesterin. However, the same intensification of reaction is usually obtained with the opaque emulsion (secured by thorough mixing) of uncholesterinized antigen, although the physical state of dispersion may not be the same in both instances. The following protocol will serve to illustrate the point:

TABLE VII

Syphilitic serum (56° C.) c.c.	Acetone insoluble lipoids		Acetone-insoluble lipoids + cholesterin	
	Rapid mixing (opalescent)	Thorough mixing (opaque)	Rapid mixing	Thorough mixing
0.2	+ + + +	+ + + +	+ + + +	+ + + +
0.1	+ +	+ + + +	+ + + +	+ + + +
0.05	+	+ + + +	+ + + +	+ + + +
0.03	−	+ + + +	+ + + +	+ + + +
0.02	−	+ +	+ +	+ +
0.01	−	+	+	+
0.005	−	−	−	−

It is only occasionally that cholesterinized antigen gives a somewhat stronger reaction than the opaque emulsion of the same antigen without cholesterin. On the other hand, we have found that cholesterinized acetone-insoluble lipoidal antigen sometimes gives a nonspecific positive reaction when used for testing unheated human sera, while the opaque emulsion without cholesterin has never caused a nonspecific fixation. It is evident, therefore, that cholesterinized antigen should not be employed for the examina-

tion of unheated human serum. For such purpose
only the opaque emulsion of acetone-insoluble lipoids
(without the addition of cholesterin) is recommended.

TECHNIQUE OF COMPLEMENT FIXATION TEST BY THE ANTIHUMAN HEMOLYTIC SYSTEM

Aside from the reagents, the following special
apparatus will be needed: Pipettes of 0.1 c.c. capac-
ity graduated to 0.01 c.c., one pipette being needed
for each specimen of active serum (0.02 c.c.); these
are not necessary if inactivated serum is used; sev-
eral 10 c.c. pipettes graduated to 0.1 c.c.; many 1 c.c.
pipettes graduated to 0.01 c.c.; small test-tubes, the
best dimension being 10 × 1 cm. (two tubes will be
required for each test and six tubes for controls in
each series of tests); larger test-tubes for complement,
antigen or amboceptor; very small flasks for mixing
the blood suspension; larger flasks or bottles as con-
tainers of physiological salt solution; thin glass tub-
ing about 4 mm. in bore for making capillary pipettes;
a test-tube rack with two parallel rows of holes.
A hot-air thermostat or, better, a water-bath, will be
needed for incubation. A convenient form of water-
bath, designed particularly for use in the Wasser-
mann reaction, is now on the market.

In handling the preparations and glassware
absolute asepsis is not required, but it is well to be
reasonably clean, bacteriologically speaking. Physio-
logical salt solution (0.9 per cent) should have been

boiled and cooled before use. Glassware should be
thoroughly rinsed with boiling water and allowed
to dry without wiping. Chemical cleanliness is
essential. The erythrocyte is a delicate cell which is
very easily destroyed or altered by many chemical
substances in small amounts. Those which are most
apt to be encountered ordinarily are soaps, weak
solutions of mineral acids and caustic or carbonated
alkalies, and bichloride of mercury. Test-tubes which
have been in contact with any of these substances
must be thoroughly washed and rinsed in clear run-
ning water, finally being boiled in pure water and
dried previous to use in the test. They should
be heated to 200° c. in a dry air sterilizer before
use.

Direct preparation for making the test includes
the following procedures:

COLLECTION OF SERUM

The amount of serum required for making the
complement fixation test varies according as the
test is to be carried out with unheated (so-called
active) or with inactivated (heated in a water-bath
for thirty minutes at 55–56° c.) serum. Approxi-
mately five times as much is required of the latter as
of the former. If the serum is to be inactivated,
5–10 c.c. of blood are drawn from the median basilic
vein of the patient with a sterile syringe or a McRae

needle (Fig. 7): When the clot has formed, it is sepa-
rated from the wall of the tube and left at room
temperature for several hours, when a clear serum

FIG. 7.   McRae needle fitted into sterile
test-tube ready for use. The blunt exit is
connected with rubber tubing for the
purpose of applying aspiration, and the
needle is inserted into the median basilic
vein.

FIG. 8.   Keidel
tube.

will exude from the clot. If the serum is not to be
used immediately, it should be kept in the
refrigerator.

Withdrawal of blood from the median basilic
vein is more easily accomplished with a sterile syringe

than with Wright capsules. Keidel has devised a sealed vacuum glass bulb connected by rubber tubing with a needle. When the needle (sterilized in the autoclave) has been inserted into the vein, the sealed end of the bulb is broken off. The blood then rushes into the bulb (Fig. 8).

If the serum is to be used while active only 1 or 2 c.c. of the patient's blood is needed, and this may be obtained conveniently by puncturing the inner side of the last joint of the middle finger with a sterile Hagedorn needle. Before puncturing compress the finger tightly in such a way as to drive the blood towards the extremity. (In small children it is more convenient to puncture the lobe of the ear.) Wright's capsules are best suited for collecting the blood. In order to get *enough* blood it is usually necessary to massage the finger towards the tip repeatedly. One puncture usually suffices. After sufficient blood is collected the straight, empty, capillary end of the tube is sealed with a flame. When cool, the capsule is shaken to drive the blood from the bent to the straight end; the bent end can then be sealed with a flame in turn. By this means the capsule can be sealed without applying the heat to the blood. Wright's capsules may be made by drawing out ordinary thin glass tubing in the flame of a good-sized alcohol lamp, or, better, a Bunsen burner.

The blood clot and the serum separate in a few hours at room temperature. If the test is not to be made within two or three days the serum should be drawn off with a capillary pipette. If left in contact with the clot it will finally become tinged with hemoglobin, which will subsequently interfere with the accurate reading of the test.

PREPARATION OF THE CORPUSCLE SUSPENSION

The suspension can be prepared with the blood of the patient being examined, more blood being drawn than is required for the actual test. The standard amount of corpuscle suspension for the writer's system is 0.1 c.c. of a 10 per cent suspension for each tube. Hence, if 1 c.c. of blood is drawn from any one of the patients it will provide enough corpuscles to distribute into 100 test-tubes. The most important condition, and one which must be strictly observed in utilizing the corpuscles of other patients, is the complete removal of serum from the suspension. This is easily accomplished by washing the blood by means of centrifugation. Two different procedures for making the suspension follow.

Fill a graduated centrifuge tube (capacity 10 c.c.) with sodium citrate solution[1] up to 9 c.c. Allow the blood of the patient to drop until it fills the tube

[1] This is prepared by adding 20 gm. of sodium citrate to 1000 c.c. of 0.9 per cent salt solution.

up to 10 c.c. mark (9 c.c. of citrate solution + 1 c.c. blood). Mix the blood well with the citrate solution and centrifuge. Pour off the supernatant fluid (containing the serum) and fill up to 10 c.c. with a fresh lot of salt solution. Stir and centrifuge again. The supernatant fluid is once more decanted off. The deposit (corpuscles free from serum) is now resuspended in 10 c.c., making a 10 per cent suspension. In the test one uses 0.1 c.c. of the 10 per cent suspension for each tube.

With defibrinated blood the suspension may be similarly made after removing the serum by washing with a large amount of salt solution. In this case also 1 c.c. of the defibrinated blood will provide enough suspension for 100 tubes.

THE TEST PROPER

The rack containing two rows of holes for the small test-tubes as shown in the illustration on p. 90 will facilitate the carrying out of the test. For each test two tubes are required, one in the front row and its control in the back row. There will also be three pairs of tubes to serve as positive, negative, and hemolytic system controls.

Put into each of two tubes, front and back, 0.02 c.c. of the active serum, or 0.1 c.c. of inactivated serum. In the case of cerebrospinal fluid, 0.2 c.c. is used. Add to each tube 0.1 c.c. of 40 per cent fresh

guinea-pig serum made by adding 1 part of comple-
ment to 1½ parts of 0.9 per cent salt solution. (See
pp. 60–61.) To the front tube add 0.1 c.c. of the
antigen in form of an emulsion, then to both tubes
add 1 c.c. of salt solution.

With every series of tests it is necessary to have
three pairs of controls as already stated (p. 89), and for
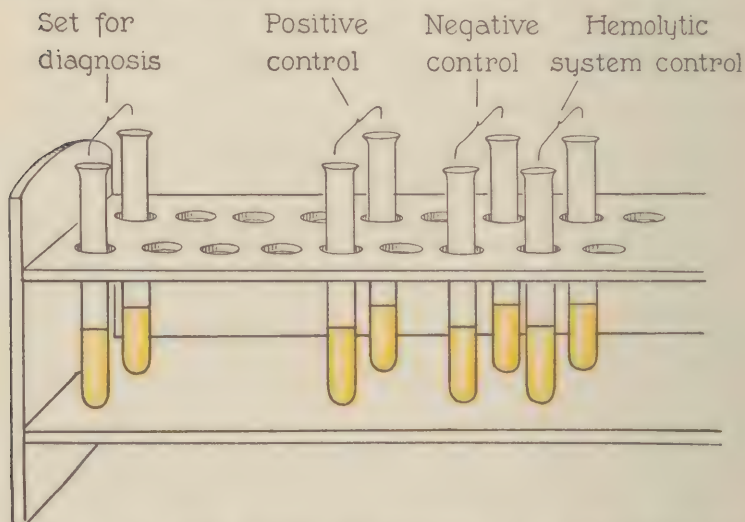
FIG. 9. The picture shows the appearance of all tubes before the
second incubation. Thus far there is no visible difference and the corpuscles
are still intact. All front tubes contain antigen.

this purpose six additional tubes will be necessary. To
each of the first pair of these, one in the front and
one in the back row, is added 0.02 c.c. of active or
0.1 c.c. of inactivated syphilitic serum known to
give a positive reaction. This will serve as a positive
control. To the second pair should be added a similar

quantity of non-syphilitic serum known to give a negative reaction. This pair of tubes will serve as a negative control. The third pair of tubes receives no serum and will be used to control the action of the hemolytic system with (front tube) and without
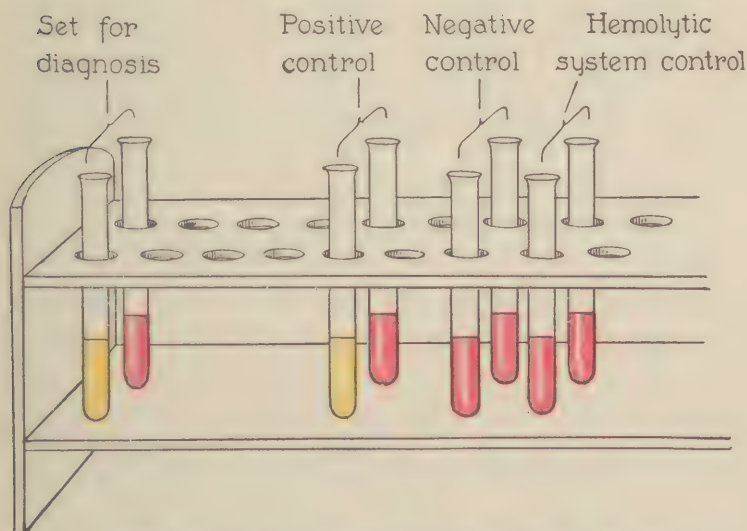
FIG. 10. This picture shows the appearance of the tubes after the second incubation, the contents having been shaken. The front tubes contain both antigen and amboceptor, the rear tubes only amboceptor. In the negative control set hemolysis occurred in both tubes. In the positive control set hemolysis took place in the back tube only and not in the front. In the set for diagnosis the conditions are seen to be identical with the positive control set, hence this serum is syphilitic.

(back tube) the antigen. Antigen is now added to the front row of control tubes and complement to all. Finally, 1 c.c. of salt solution is put into each tube. Shake the tubes thoroughly to distribute the reagents evenly through the mixtures.

The rack holding the tubes is incubated at 37° c. in a water-bath for thirty minutes or in an air thermostat for one hour. During this time the antibody combines with the antigen and complement is fixed. At the end of the incubation period add to all the tubes 0.1 c.c. of the 10 per cent corpuscle suspension and 0.1 c.c. of liquid antihuman amboceptor corresponding to 2 units and repeat the incubation. The purpose of the second incubation is to allow the hemolytic system to act.

During the second incubation of one hour in the thermostat or thirty minutes in the water-bath the tubes must be given gentle but thorough shakings at intervals to secure a uniform reaction of the hemolytic system, because the corpuscles, unless occasionally stirred up, will gradually settle to the bottom and escape the full effect of the hemolysin. After this final incubation the tubes should be kept at room temperature for one hour before the results are recorded.

The entire procedure is given in the charts. For the sake of clearness the procedures in the case of active and inactivated serum are given in separate charts (Charts III and IV). In case paper amboceptor is used (Chart V), the amboceptor slips had best be added before the first incubation to give them time to dissolve. At the end of the incubation period 0.1 c.c. of the 10 per cent corpuscle suspension is added, as shown in Chart III.

Before reading the results, it is necessary to make certain that the tests in the control sets have been properly carried out. The pairs of tubes containing negative serum and those without serum must be
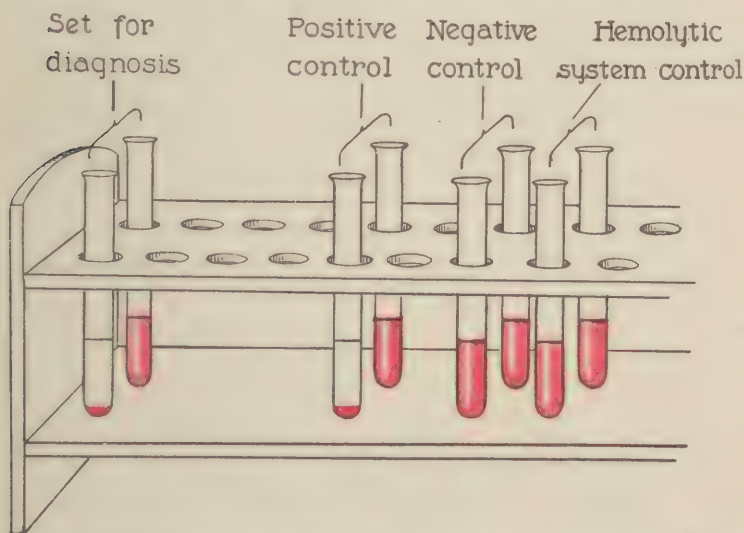
Fig. 11. This picture shows the appearance of the tubes shown in Fig. 10 after they have stood one hour. The absence of hemolysis in the front tubes of the positive control and diagnostic sets is shown by the clear supernatant salt solution over the deposited intact corpuscles. The absence of hemolysis means a positive reaction in these instances.

completely hemolyzed. These constitute the negative controls and show that the hemolytic system used is effective and that the amount of antigen used does not of itself inhibit hemolysis.

The front tube of the positive control set, containing a known syphilitic serum, must show total inhibition of hemolysis, while the back tube must show complete hemolysis. We are then certain that

CHART III

PROCEDURE FOR EXAMINATION OF ACTIVE (UNHEATED) SERUM

First incubation at 37° C. for thirty minutes in the water-bath or one hour in an air thermostat

Addition of 0.1 c.c. of 10 per cent corpuscle suspension and 0.1 c.c. (2 units) of antihuman amboceptor

Second incubation at 37° C. for the same length of time as the first

Reading of results after one hour at room temperature

	Set for diagnosis Test of serum in question	Positive control set (with known syphilitic serum)	Negative control set (with known non-syphilitic serum)	Hemolytic system control set (without serum)
Back row	Unknown serum (unheated)*.... 0.02 c.c. 40 per cent complement...... 0.1 c.c. 0.9 per cent salt solution...... 1.0 c.c.	Known syphilitic serum (unheated)...... 0.02 c.c. 40 per cent complement...... 0.1 c.c. 0.9 per cent salt solution...... 1.0 c.c.	Known negative serum (unheated)...... 0.02 c.c. 40 per cent complement...... 0.1 c.c. 0.9 per cent salt solution...... 1.0 c.c.	No serum. 40 per cent complement...... 0.1 c.c. 0.9 per cent salt solution...... 1.0 c.c.
Front row	Same as above plus antigen... 0.1 c.c.	Same as above plus antigen... 0.1 c.c.	Same as above plus antigen... 0.1 c.c.	Same as above plus antigen... 0.1 c.c.

* In the case of cerebrospinal fluid, 0.2 c.c. is used.

CHART IV

PROCEDURE FOR THE EXAMINATION OF INACTIVATED SERUM

First incubation at 37° C. for thirty minutes in the water-bath or one hour in an air thermostat

Addition of 0.1 c.c. of 10 per cent corpuscle suspension and 0.1 c.c. (2 units) of antihuman amboceptor

Second incubation at 37° C. for the same length of time as the first

Reading of results after one hour at room temperature

	Set for diagnosis Test of serum in question	Positive control set (with known syphilitic serum)	Negative control set (with known non-syphilitic serum)	Hemolytic system control set (without serum)
Back row	Unknown serum (56° C.)........ 0.1 c.c. 40 per cent complement........ 0.1 c.c. 0.9 per cent salt solution........ 1.0 c.c.	Known syphilitic serum (56° C.).. 0.1 c.c. 40 per cent complement........ 0.1 c.c. 0.9 per cent salt solution........ 1.0 c.c.	Known negative serum (56° C.).. 0.1 c.c. 40 per cent complement........ 0.1 c.c. 0.9 per cent salt solution........ 1.0 c.c.	No serum. 40 per cent complement........ 0.1 c.c. 0.9 per cent salt solution........ 1.0 c.c.
Front row	Same as above plus antigen.... 0.1 c.c.	Same as above plus antigen.... 0.1 c.c.	Same as above plus antigen.... 0.1 c.c.	Same as above plus antigen.... 0.1 c.c.

CHART V

PROCEDURE FOR THE USE OF PAPER AMBOCEPTOR

First incubation at 37° C. for thirty minutes in the water-bath or one hour in an air thermostat.

Addition of 0.1 c.c. of 10 per cent corpuscle suspension.

Second incubation at 37° C. for the same length of time as the first.

Reading of results after one hour at room temperature.

	Set for diagnosis Test of serum in question	Positive control set (with known syphilitic serum)	Negative control set (with known non-syphilitic serum)	Hemolytic system control set (without serum)
Back row	Unknown serum.* 40 per cent complement........ 0.1 c.c. 0.9 per cent salt solution........ 1.0 c.c. Amboceptor paper, 2 units	Known syphilitic serum.* 40 per cent complement........ 0.1 c.c. 0.9 per cent salt solution........ 1.0 c.c. Amboceptor paper, 2 units	Negative serum.* 40 per cent complement........ 0.1 c.c. 0.9 per cent salt solution........ 1.0 c.c. Amboceptor paper, 2 units	No serum. 40 per cent complement........ 0.1 c.c. 0.9 per cent salt solution........ 1.0 c.c. Amboceptor paper, 2 units
Front row	Same as above plus antigen..... 0.1 c.c.	Same as above plus antigen..... 0.1 c.c.	Same as above plus antigen..... 0.1 c.c.	Same as above plus antigen.... 0.1 c.c.

* 0.02 c.c. of active (unheated) or 0.1 c.c. of inactivated (56° C.) serum; 0.2 c.c. of cerebrospinal fluid.

the syphilitic serum itself does not inhibit hemolysis, and that the syphilitic antibody is able to fix complement in the presence of the antigen employed.

These essentials having been fulfilled, the tubes containing serum for diagnosis can be examined. In these tests hemolysis must be complete in the back tube if the serum used is not anticomplementary, since antigen is not present (Fig. 11, extreme left, rear tube).

In the front tube, containing the serum for diagnosis and antigen, any degree of hemolysis may be encountered, from complete dissolution of corpuscles to total absence of hemolysis, depending on the presence or absence of syphilitic antibodies. With complete inhibition of hemolysis the reaction is easily interpreted, the corpuscles settling to the bottom of the tube with the clear salt solution above (Fig. 11, the front tube of the pair at extreme left). Complete hemolysis likewise gives a result easy of interpretation, for the corpuscle mass is entirely dissolved, the hemoglobin going into solution and coloring the salt solution a deep reddish color (Fig. 11, the back tube of the positive control, and all tubes of the negative and hemolytic system control sets).

Interpretation of Results. By taking into consideration the bulk of corpuscles settling to the bottom of the tube and the amount of tinting of the supernatant salt solution, and by comparison with the positive and negative controls, the varying degrees

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FIG. 12.

of inhibition of hemolysis may be ascertained (Fig.
12). In interpreting the result complete inhibition
of hemolysis comparable with the positive control
is called strongly *positive* and is arbitrarily designated
++++; complete hemolysis, comparable with the
negative control, is designated as a *negative reaction.*
If more than 90 per cent of the bulk of corpuscles
is dissolved the reaction is *doubtful* (±) and should
not be taken into consideration for diagnosis. In
a known specific case such slight inhibition should
be an indication for further treatment as evidence
of the continued activity of syphilitic infection. If
there is no hemolysis, or only a faint trace of it
present, the reaction may be classed as *strongly
positive.* A more intense hemolysis, less than 25 per
cent dissolution of the corpuscle mass, may be called
positive (+++), 50 per cent hemolysis may be termed
weakly positive (++), while 75 per cent hemolysis is
designated as *faintly positive* (+). *Neither the very
weakly positive nor the faintly positive reaction should
be accepted as a definite diagnosis of syphilis without
the presence of strong clinical evidence in favor of such
a diagnosis.*

In case the reaction should be doubtful, the serum
may be re-examined after a period of a week has
elapsed, and if the reaction is still indecisive several
examinations in succession may be made. The reac-
tion may sometimes be very weak in a case of undis-

puted syphilis. In such an instance the test should be repeated not only with 0.02 c.c. of the active or 0.1 c.c. of the inactivated but at the same time with 0.04 c.c. of the active or 0.2 c.c. of the inactive serum. Very often a definite positive reaction can be obtained with the larger quantity. For examining the sera from known cases of syphilis for prognostic purposes the test must be made with 0.04 c.c. of the active or 0.2 c.c. of the inactivated serum in case the routine amount no longer gives a positive reaction. In all cases it is a wise precaution to take the blood for examination shortly before meal-time, and, as pointed out by Craig and Nichols, the patient should be denied alcohol for twenty-four hours previous to the taking of blood.

It may be stated here that certain specimens of human sera gradually become anticomplementary on standing several days, some more so than others. This change sets in much more rapidly at a higher temperature (that of a room) than at a lower temperature (refrigerator).

Whenever examining an unknown serum one must always have at hand a syphilitic serum known to give a positive reaction as a positive control. If a good specimen is obtained it may be preserved on ice for months. Should it become too anticomplementary on standing, this property can be removed

by heating the serum at 55°–56° C. for about fifteen minutes.

In the routine examination of a patient's serum for the presence of syphilitic antibodies, as previously stated, 0.02 c.c. of the active or 0.1 c.c. of the inactivated serum is used, that is, this amount of serum is used to determine whether or not the serum is that of a luetic case. When a strong positive reaction is obtained in several specimens we are unable to distinguish the intensity of the reaction without further analysis. In fact, a positive reaction may be secured with any syphilitic serum containing more than one unit of syphilitic antibody, and in order to determine the exact strength of each specimen, it may be titrated as follows:

Prepare a 1:50 dilution of the fresh serum by mixing 0.1 c.c. with 4.9 c.c. of salt solution. This dilution is of such strength that 1 c.c. contains 0.02 c.c. of the original serum. In the case of inactivated serum a 1:10 dilution (0.5 c.c. serum plus 4.5 c.c. of salt solution) will correspond to 0.1 c.c. of the undiluted serum. 1 c.c. of the dilution is the maximum volume of fluid for one tube and contains the maximum amount of the serum chosen for the test. To titrate

the strength of a fresh serum giving a positive reaction in the amount of 0.02 c.c. we have to test different amounts of the same serum below 0.02 c.c. For accomplishing this end graded quantities of the diluted serum are measured into a number of tubes, as shown in the following protocol:

TABLE VIII

AMOUNT OF DILUTED SERUM

Tube						c.c. inactivated serum)
1......	1 c.c.	(equal to 0.02 c.c. active	"	0.1		
2......	0.5	" " 0.01	"	0.05	"	"
3......	0.4	" " 0.008	"	0.04	"	"
4......	0.3	" " 0.006	"	0.03	"	"
5......	0.25	" " 0.005	"	0.025	"	"
6......	0.2	" " 0.004	"	0.02	"	"
7......	0.15	" " 0.0028	"	0.015	"	"
8......	0.1	" " 0.002	"	0.001	"	"

Having measured the amounts of the serum into the tubes and brought the total volume of fluid of each tube up to 1 c.c. uniformly by adding salt solution, one now makes the fixation test in the usual way. Add to each tube 0.1 c.c. of 40 per cent dilution of guinea-pig's complement, 0.1 c.c. of the standard antigen emulsion. Mix the contents well by shaking and incubate the tubes at 37° C. for 1 hour in the air thermostat or 30 minutes in the water-bath. At the end of the incubation add to each tube 0.1 c.c. of 10 per cent suspension of washed human corpuscles and 2 units of amboceptor, and repeat the incubation. If a given specimen gives complete fixation only in the first tube (1 c.c.), it is said to contain only one unit of antibody. If it occurs also in

the second tube (0.5 c.c.) it contains two units; in
the third (0.4 c.c.), 2.5 units; in the fourth (0.3 c.c.),
3.3 units, etc. A specimen showing complete fixation
in the last tube (0.1 c.c.) must contain at least ten
antibody units and may be titrated with another
tenfold dilution in order to find out its real titre.

By this means the titre of any specimen can easily
be determined. The writer has examined specimens
which contained as many as 30 units in 0.02 c.c. of
serum.

In titrating cerebrospinal fluid one must prepare
the stock dilution by mixing 1 c.c. of it with 4 c.c.
of salt solution. The process of titration is exactly
the same as that described for the serum.

PRECAUTIONS TO BE OBSERVED

The preliminary adjustment of the hemolytic sys-
tem is essential to obtaining reliable results (pp. 31–
35, 64–66). If previous determination is made of the
minimum quantity of amboceptor which will hemolyze
0.1 c.c. of a 10 per cent corpuscle suspension in the
presence of 0.1 c.c. of 40 per cent guinea-pig comple-
ment by the end of 30 minutes, and twice this
quantity of amboceptor is used in the test, hemolysis
will proceed at the normal rate. Too rapid hemolysis
is apt to mask a positive reaction, and incomplete
hemolysis may stimulate positive reactions.

The corpuscles should be as fresh as possible when used. They decrease rapidly in resistance after the seventy-two-hour period, and more quickly the higher the temperature. If old corpuscles are used, they may deteriorate so rapidly that a titration of an hour previously will no longer determine how they will behave in the test. Complement, owing to its great lability, is likely to deteriorate. Hence it is good practice to employ complement that is not older than forty-eight hours (preferably under twenty-four hours), and has been kept constantly at refrigerator temperature. The attempt should not be made to utilize deteriorated complement in larger quantities, because such a specimen does not give a reliable reaction. Guinea-pig's complement may sometimes remain unfixed and mask the positive reaction. It is always best to use a mixture of the sera from two or more guinea-pigs.

In testing several specimens of active serum at one time it occasionally happens that the hemolytic reaction is completed more slowly in some than in others. The cause of this slowness is not present in the complement or amboceptor, but in the sera themselves, which contain anticomplementary substances. To remove this source of error, it is necessary *to heat such sera to 55° C. for twenty minutes and use 0.1 c.c. for the test.* Inactivated sera are rarely anticomplementary. The difficulty may be obviated

in some cases by collecting the serum to be tested just before meal-time, because the anticomplementary substance is closely associated with the absorption of the chyle into the circulation soon after the meal.

The quality and quantity of the antigen may also be sources of error, hence the necessity of careful and frequent titration of antigen (see pp. 74–78).

If it is desired to make the test with larger quantities, one has simply to multiply the quantity of each factor employed; for example, one may use 0.1 c.c. of the complement, 0.05 c.c. of the patient's serum, 1 c.c. of a 5 per cent suspension of the washed human corpuscles, and 2 units of the amboceptor (titrated with the above complement and corpuscle-suspension unit), in a total volume of 5 c.c. of physiological salt solution. This increase in the relative quantities of each constituent offers one advantage and one disadvantage. The advantage is that the intensity of the reaction can be more minutely measured through the liberation of the hemoglobin, as the number of red corpuscles is, of course, much larger. The disadvantage arises from the unnecessary waste of material. For the worker in a regularly equipped biological laboratory this waste may make but little difference, but for those who intend to do the test in a private laboratory the exercise of economy is highly desirable.

When the serum to be tested has previously been inactivated by being heated to 56° c., the amount of serum used must be from four to five times greater than that prescribed for the fresh or unheated serum, since one effect of the inactivation is to reduce the content of the antibody to about one-fourth or one-fifth of the original strength. The effect of inactivation upon the antibody content of serum has bearing not only on what has already been said, but with equal if not greater directness upon the original Wassermann and other systems requiring inactivation of the patient's serum (see Chapter VII).

CHAPTER IX

Homohemolytic Systems for the Serum Diagnosis of Syphilis

UNDER ordinary circumstances the antihuman hemolytic system, with guinea-pig complement, is most satisfactory, but in emergencies in which guinea-pig serum is not available, a reliable diagnosis can be made by substituting human for guinea-pig complement. In certain exceptional cases, in which the complement content of the serum is above or below the average, special adjustment of the hemolytic system must be made. Even this disadvantage, however, is removed by the use of a definite amount of human complement with inactivated serum, as shown by Butler and Landon (p. 109).

Efforts to utilize human complement have been made by Tschernogubow, Emery, Butler and Landon, Myer, and Thompson.

Tschernogubow proposed the use of a suspension of the patient's blood directly diluted in saline solution in a ratio of 1 drop to 1 c.c. of saline. This was to serve as the source of complement, corpuscles, and, if present, the fixing substance. By careful scrutiny, however, an enormous disproportion is discovered among the various elements concerned. For example, the amount of serum probably present in

1 drop of blood, which would perhaps be 0.07 c.c.,
would be approximately 0.035 c.c. at most, while
the corpuscular suspension approaches 7 per cent.
The amount of syphilitic antibodies present would
be too small to make possible the detection of a
weak positive reaction, and the minuteness of com-
plement present in such a mixture precludes any
practical possibility. Even an enormous amount of
the amboceptor fails to complete hemolysis of such
a concentrated suspension of the corpuscles. More-
over, the mixture forms a loose gelatinous fibrin,
involving the whole contents, making it impossible
to stir by shaking. When the first fibrin is removed,
a second may form on further standing. Such a
method cannot be used, and Tschernogubow him-
self soon abandoned it.

Although the first attempt of Tschernogubow
failed to develop into a practical success, the ques-
tion of utilizing human complement in the Wasser-
mann reaction has since attracted the attention of
several investigators, who in turn have proposed
various procedures in which the quantitative adjust-
ment of different reagents is more carefully made.
Emery, Butler and Landon, and Thompson have
worked out such methods.

Emery employs active serum with the cholester-
inized alcoholic extract of heart muscle, disregarding
the possibility of obtaining a false positive fixation

with certain non-syphilitic sera. The amount of the patient's serum is minute, but the concentration of the corpuscles is disproportionately large (20 per cent of the firmly packed sediment after centrifugation). Wright's capillary technique is used.

The method of Butler and Landon in its hemolytic system seems to be nearly ideal.

Inactivated serum and human complement are used in combination with the writer's acetone-insoluble antigen. The only possible disadvantage of the procedure is the necessity of obtaining sufficient normal human serum as complement in the event that a large number of tests are made daily. Butler and Landon also use Wright's capillary method. Small glass tubes, 6 by 0.6 cm., are used, and the final quantity in each tube is 0.4 c.c.

Complement: Blood is drawn from the vein of a non-syphilitic person, placed in the incubator for 1 hour, in the ice box over night, centrifuged in the morning and the serum withdrawn.

Amboceptor: The amboceptor (paper) is titrated with 0.02 c.c. of a known negative human serum as complement and 0.02 c.c. of a $7\frac{1}{2}$ per cent human red blood cell suspension, the least amount of amboceptor which causes complete hemolysis by the end of 30 minutes being taken as 1 unit. Two units are used in the test.

Blood cell suspension: Blood is drawn from a vein into sodium citrate solution, washed twice in 0.9 per cent salt solution, and a $7\frac{1}{2}$ per cent suspension in 0.9 per cent salt solution used in the test.

The cells are sensitized before being added to the tubes by adding amboceptor paper to a $7\frac{1}{2}$ per cent cell suspension in a ratio of 2 units to 0.16 c.c. of cell suspension, the amount of

cells sensitized being calculated to give a sufficient supply for the occasion. To economize time the sensitization of cells is begun first, the fixation test separately, and after an incubation of 1 hour for the former and 30 minutes for the latter, 0.16 c.c. of the sensitized cells is added to each tube of the fixation series. After an incubation of 30 minutes in the water bath the test is completed. If sensitization is sufficient, hemolysis in the control tubes is complete after about five minutes.

The determinative tube receives 0.02 c.c. human complement, 0.14, c.c. of a 1:14 dilution of antigen, 0.08 c.c. inactivated serum of patient, 0.16 c.c. sensitized human red cells; the control tube receives 0.02 c.c. human complement, 0.14 c.c. salt solution, 0.08 c.c. inactivated serum of patient, and 0.16 c.c. of sensitized human red cells.

Thompson (1917) proposed to utilize the complement of the fresh serum. His preliminary titration of the complement activity of each specimen and adjustment adds extra labor to the test but eliminates any possibility of quantitative error. He also uses the acetone-insoluble antigen.

Preparation of Reagents. The details with regard to the reagents used in conducting the writer's homohemolytic system are essentially the same as those described in connection with the antihuman heterohemolytic system (pp. 60–84).

VARIETIES OF IRREGULAR REACTIONS AND THEIR ADJUSTMENT

Reference has already been made to the possible deficiency of complement in certain specimens of

fresh human sera. With these hypocomplementary sera hemolysis in the control tubes without antigen proceeds slowly and remains incomplete at the end of the second incubation. To these sets of tubes, both the determinative and the control, an additional hemolytic amboceptor unit may be introduced in order to reinforce the hemolytic activity of the complement. In cases in which hemolysis is nearly complete only one-half the unit is added, but when hemolysis is slight a whole unit of the amboceptor is needed. The tubes should then be put in a separate rack and subjected to further incubation until hemolysis is complete in the control tubes (without the antigen). Fifteen minutes or longer may be required. Specimens which still fail to hemolyze completely in the control tube, even with the additional amboceptor, should be tested again by adding to them a quantity of fresh serum which has been shown to contain an average complement and at the same time to be devoid of any syphilitic fixation substance (negative serum). In these cases 0.1 c.c. of fresh serum serving as complement is used with 0.2 c.c. of the serum deficient in complement. The mixture is then tested in the same way as any fresh serum.

POSITIVE AND NEGATIVE CONTROLS

As in any other serodiagnostic procedure, each serum tested must be accompanied by a positive and

a negative serum in order to control the reliability of the reagents. In a well appointed laboratory, where many tests are being made daily or every other day, the necessary positive and negative sera will be furnished by the tests of the previous occasion.

PRINCIPLE OF THE METHOD

It is well known that complement is present in every fresh serum and that the quantity may sometimes vary. In the serodiagnosis of syphilis, whether by the antisheep system of Wassermann or the antihuman system of the writer, the serum of the guinea-pig is chosen because of its richness in complement and also because guinea-pig complement is probably more readily fixed by the antigen-antibody combination than the sera of other animals, such as the horse, rabbit, sheep, pig, etc. As to the complement in fresh human sera, there are not many data regarding its action upon human corpuscles. It is understood that human sera exert no hemolytic action upon human corpuscles, especially when there is no iso-hemolysin (see pp. 39–40). But upon the addition of a sufficient quantity of the antihuman hemolytic amboceptor the complement dissolves the human corpuscles just as guinea-pig complement does when added to the suspension of human corpuscles and the specific antihuman hemolytic amboceptor.

The only difference between the action of the human and the guinea-pig complement lies in the fact that the former requires more anti-human hemolytic amboceptor to render it active against the human corpuscles than the latter. The relative lytic values of the human and the guinea-pig complement are shown in the following experiments (Tables IX and X).

TABLE IX

TITRATION OF THE ANTIHUMAN AMBOCEPTOR WITH GUINEA-PIG COMPLEMENT

Amount of amboceptor*	Amboceptor No. 633 (rabbit)		Amboceptor No. 634 (rabbit)	
c.c.				
0.01	Complete hemolysis.		Complete hemolysis.	
0.007	"	"	"	"
0.005	"	"	"	"
0.004	"	"	"	"
0.003	"	"	"	"
0.002	"	"	"	"
0.0015	"	"	"	"
0.001	"	"	"	"
0.0007	"	"	"	"
0.0005	Partial hemolysis.		"	"
0.0004	No	"	Partial	"
0.0003	"	"	No	"
0.0002	"	"	"	"
No serum	"	"	"	"

* Each tube contained guinea-pig complement 0.04 c.c. and 1 per cent human corpuscle suspension 1 c.c.

TABLE X

TITRATION OF THE ANTIHUMAN AMBOCEPTOR WITH HUMAN COMPLEMENT

Human complement + amboceptor*	Result
c.c. c.c.	
0.1 + 0.1	Complete hemolysis.
0.1 + 0.05	" "
0.1 + 0.03	" "
0.1 + 0.02	" "
0.1 + 0.01	" "
0.1 + 0.005	" "
0.1 + 0.003	Considerable "
0.1 + 0.002	No "
0.1 + 0.001	" "
0.5 + 0.02	Complete hemolysis.
0.3 + 0.02	" "
0.2 + 0.02	" "
0.1 + 0.02	" "
0.05 + 0.02	Slight hemolysis.
0.02 + 0.02	No "

*Human serum as complement. 1 per cent human corpuscle suspension
1 c.c. in each tube.

It is evident that the amount of amboceptor
required to dissolve the human corpuscles in the
presence of human complement is many times that
necessary with guinea-pig complement.

ADJUSTMENT OF HEMOLYTIC SYSTEM

In carrying out the homohemolytic system it is
necessary to find the minimum hemolytic dose of
antihuman amboceptor which will cause complete

hemolysis in the presence of 0.2 c.c. of fresh human serum by the end of fifteen minutes at 37° c. (water-bath). This quantity is to be regarded as 1 unit of amboceptor and is employed in conjunction with 0.2 c.c. of the human serum. When the amount of amboceptor representing 1 unit has been determined it is best to make a dilution such that 0.1 c.c. of the dilution will contain 1 unit. The corpuscle suspension is the same as that used in the writer's original system. (See pp. 64–66 for adjustment of hemolytic system.)

PROCEDURE FOR THE EXAMINATION OF HUMAN SERA NOT MORE THAN FORTY-EIGHT HOURS OLD

It must be made clear in the beginning that specimens should be examined as soon as practicable, preferably within twenty-four hours after they are withdrawn from the patients. After forty-eight hours, even at the temperature of the refrigerator, the complement gradually disappears from the serum. Sera kept for more than three days in a refrigerator must be tested by a special technique, to be given later. Specimens tinged deeply with hemoglobin give unsatisfactory results and should be rejected. Table XI indicates the amounts of reagents to be used in the test for fresh human sera and other details regarding it. Each tube should be accompanied by a control tube without antigen.

TABLE XI

PROCEDURE FOR EXAMINING FRESH HUMAN SERA

Tube	1st step	2nd step	3rd step	4th step	Final step
Determinative tube (front row)	Patient's serum (fresh).. 0.2 c.c. Antigen.............. 0.1 c.c. 0.9 per cent saline solution............... 1.0 c.c.	First incubation at 37° C. for thirty minutes in water bath or one hour in air thermostat	Both tubes receive 0.1 c.c. of antihuman amboceptor, representing 1 hemolytic unit (15 minutes), and 0.1 c.c. of 10 per cent human corpuscular suspension. Total volume 1.5 c.c. Contents are well mixed by shaking	Second incubation at 37° C., same as first, except that tubes are shaken three times during the period	Reading of results after tubes have stood thirty minutes at room temperature
Control tube (back row)	Patient's serum (fresh).. 0.2 c.c. Antigen omitted. 0.9 per cent saline solution............... 1.1 c.c.				

PROCEDURE FOR THE EXAMINATION OF HUMAN SERUM
MORE THAN FORTY-EIGHT HOURS OLD

As previously stated, specimens of human serum which have stood in a refrigerator longer than forty-eight hours are inconstant in their complementary activity, and many are markedly deficient. As a rule sera not more than seventy-two hours old which have been kept constantly in the refrigerator at 4–6° c. still contain enough complement to make the test possible. It is best, however, to inactivate all sera whose complementary activity is no longer a certain factor, and to test them with negative fresh sera containing active complement. 0.2 c.c. of the complement serum is added to 0.2 c.c. of the inactivated serum and the mixture then tested like any fresh serum. Table XII gives the details of the procedure.

Procedure for the Examination of Cerebrospinal Fluids. This procedure is comparatively easy and gives an entirely satisfactory result. It differs from that used for inactivated sera only in one respect, that of the quantity of the specimen used, which may vary from 0.2 to 0.5 c.c. No inactivation is required as the cerebrospinal fluid contains no complement, and 0.2 c.c. of active negative human serum (previously tested) is added as complement (Table XIII).

TABLE XII

PROCEDURE FOR THE EXAMINATION OF INACTIVATED HUMAN SERA WITH HUMAN COMPLEMENT

Tube	1st step	2nd step	3rd step	4th step	Final step
Determinative tube (front row)	Patient's serum (inactivated)............. 0.2 c.c. Active negative serum. 0.2 c.c.* Antigen............. 0.1 c.c. 0.9 per cent saline solution............. 0.9 c.c.	First incubation for thirty minutes in water-bath or one hour in air thermostat.	Antihuman amboceptor, 1 unit in 0.1 c.c.; 10 per cent human corpuscular suspension, 0.1 c.c. Total volume 1.6 c.c. Contents are well mixed	Second incubation same as first except that tubes are shaken three times during incubation	Reading of results within thirty minutes after removal of tubes from incubator
Control tube (back row)	Patient's serum (inactivated)............. 0.2 c.c. Active negative serum. 0.2 c.c.* Antigen omitted. 0.9 per cent saline solution............. 1.0 c.c.				

* A second complete test of this serum should accompany the test of the serum to which it is added as complement.

TABLE XIII

PROCEDURE FOR THE EXAMINATION OF CEREBROSPINAL FLUID

Tube	1st step	2nd step	3rd step	4th step	Final step
Determinative tube (front row)	Cerebrospinal fluid.... 0.2 c.c.* Active negative serum. 0.2 c.c.† Antigen................. 0.1 c.c. 0.9 per cent saline solution................. 0.9 c.c.	First incubation same as in Tables XI and XII	Antihuman amboceptor, 2 units in 0.1 c.c.; 10 per cent human corpuscular suspension, 0.1 c.c. Total volume 1.6 c.c. Contents are mixed by shaking	Second incubation same as first except that contents of tubes are shaken three times during incubation period	Reading of results within thirty minutes after removal of tubes from incubator.
Control tube (back row)	Cerebrospinal fluid.... 0.2 c.c. Active negative serum. 0.2 c.c.† Antigen omitted. 0.9 per cent saline solution................. 1.0 c.c.				

* Graduated quantities of from 0.2 to 0.5 c.c. may be used in certain cases, the amount of saline solution being so adjusted as to make the total volume 1.4 c.c.

† A complete test of this serum should accompany the test of the serum to which it is added as complement.

RESULTS OF PRACTICAL APPLICATION OF THE TEST

Trials of the method were made with 1,331 specimens of blood and 52 cerebrospinal fluids. Of 1,118 specimens of sera from these sources, 517 gave a positive reaction, the results conforming to those reported by the serological departments of the various hospitals. Of 132 specimens from psychiatric cases, 54 were from general paralysis cases, and all except 2 gave a strongly positive reaction. Among other psychoses, including 75 cases of dementia præcox, 3 of alcoholic psychosis, 3 of imbecility, 3 of senile psychosis, 6 of arteriosclerosis, 1 of manic-depressive insanity, and 1 paranoic condition, there were only 2 positive reactions, these occurring among the dementia præcox cases. The reactions with 81 inactivated sera agreed with those obtained by others with the same material. Twenty cerebrospinal fluids from cases of general paralysis gave a strongly positive reaction, while 32 specimens from other non-syphilitic cases showed a negative reaction. The statement will perhaps bear repeating that of 1250 fresh human sera, complement was deficient in 93 specimens, which had to be examined either by means of additional amboceptor or by supplying active human complement from fresh negative sera. This special adjustment with fresh sera is one which demands particular attention on the part of serologists adopting the method.

NON-INTERFERENCE BY NEGATIVE SERUM IN COMPLE-
MENT FIXATION IN THE HOMOHEMOLYTIC SYSTEM

The use in the homohemolytic system of fresh nega-
tive serum as complement when there is no comple-
ment or too little in the specimen to be examined,
while entirely analogous to the use of fresh guinea-
pig serum as complement for an inactivated patient's
serum, may rouse apprehension as to the possibility
of reduction in the degree of fixation by this compara-
tively large amount of human complement, owing to
its indifferent serum constituents. The experiments
recorded in Table xiv, in which human and guinea-
pig complement were studied in parallel series, show
that the addition of an inactivated negative serum to
a syphilitic serum does not cause any so-called com-
plementoid blocking of the complement fixation of
any significance, and that no error can result from
this source.

All the other experiments of this sort with syphilitic
sera gave similar results. It was found, however, that
a syphilitic serum containing less than one-half an
antibody unit caused less inhibition in the tubes
to which more than 0.3 c.c. of the inactivated negative
human serum had been added, but never completely
masked the reaction. There was no appreciable
difference in the tubes containing 0.1 or 0.2 c.c. of
the inactivated serum and that containing none. The

TABLE XIV

EFFECT OF INACTIVATED NEGATIVE HUMAN SERUM UPON THE COMPLEMENT FIXATION

With inactivated syphilitic serum

Tube No.	With human complement	Results
1	Syphilitic serum (56° C.) containing 1 antibody unit.... 0.2 c.c. Human complement (active serum)................ 0.1 c.c.	All gave complete fixation regardless of the addition of the inactivated negative serum
2	The same + negative human serum (56° C.)........... 0.1 c.c.	
3	The same + negative human serum (56° C.)........... 0.2 c.c.	
4	The same + negative human serum (56° C.)........... 0.3 c.c.	
5	The same + negative human serum (56° C.)........... 0.4 c.c.	

With active syphilitic serum

Tube No.	With human complement	Results
1	Active syphilitic serum, containing 4 antibody units... 0.2 c.c. The same + negative human serum (56° C.)........... 0.1 c.c.	All gave complete fixation regardless of the inactivated negative serum
2	The same + negative human serum (56° C.)........... 0.2 c.c.	
3	The same + negative human serum (56° C.)........... 0.3 c.c.	
4	The same + negative human serum (56° C.)........... 0.4 c.c.	

Tube No.	With guinea-pig complement	Results
1	Syphilitic serum (56° C.) containing 1 antibody unit.... 0.2 c.c. Guinea-pig complement 40 per cent............... 0.1 c.c.	All gave complete fixation, no interference being observed from the addition of the inactivated negative human serum
2	The same + negative human serum (56° C.)........... 0.1 c.c.	
3	The same + negative human serum (56° C.)........... 0.2 c.c.	
4	The same + negative human serum (56° C.)........... 0.3 c.c.	
5	The same + negative human serum (56° C.)........... 0.4 c.c.	

addition, however, of inactivated guinea-pig serum (56° c.) to a syphilitic serum produces marked weakening of the fixation reaction, and even complete blocking when more than 0.3 c.c. of that serum is added to one syphilitic antibody unit. This confirms the earlier observations of Bronfenbrenner and the writer.

This method is undoubtedly less satisfactory than the writer's original method in which guinea-pig complement is used; nevertheless, it gives results which correspond remarkably well with those of the original method in the majority of cases. The writer has never encountered a false positive reaction by this technique, although he has found specimens of positive serum which gave a weak or negative reaction because of the excess of complement which it contained. This error can be avoided by noting carefully the rapidity of hemolysis in the control tube. If complete hemolysis takes place in five minutes it is obvious that there is an excess of complement in the specimen, and if a negative or weakly positive reaction is obtained with such a serum the test should be repeated, the amount of the serum used being reduced to such an extent that complete hemolysis takes place in the control tube in fifteen minutes. Finally, it should be emphasized that only the acetone-insoluble fraction of tissue lipoids of required standards, as set forth in

earlier pages, should be used when utilizing the human complement in the fixation test.

INFLUENCE OF TEMPERATURE UPON THE VELOCITY OF THE COMPLEMENT FIXATION REACTION

It has become customary to carry out immunity reactions *in vitro* at the temperature of 37° c., because most of the reactions between specific antibodies and antigens take place only at 37° c. or near that point. The reactions of bacteriolysis, cytolysis, hemolysis, agglutination, precipitation, phagocytosis, opsonization, etc., manifest their maximum activities at 37° c. Certain biological reactions, however do not necessarily follow this general rule. For example, hemolysis produced by saponin, bile salts, cobra lecithid, or sodium oleate is complete within a very short time whether at 4° or 37° c. The velocity of the reaction in these instances is such that time and temperature play but a slight part.

The mechanism of the lipotropic complement fixation of syphilitic serum or spinal fluid is not understood, but it is certain that the lipoids are an important factor. The question in regard to the velocity of this reaction has not received as much attention as it deserves. The prevailing idea is that it is one of the immunity reactions which manifest their maximum activity at 37° c. Whether or not the reaction can take place at lower temperatures has

not been carefully studied. The writer has been interested in this phase of the problem and has carried out numerous experiments to determine the relation between time, temperature and reaction. The determination of these points is of practical importance at the present time, since, should the reaction prove to take place satisfactorily at a temperature which can be obtained without the aid of a special incubator, the performance of the test becomes much more widely adaptable. That this is the case is shown in the following experiments.

Detection of Varying Known Quantities of the Fixing Substance ("Syphilitic Antibody") at Different Temperatures. It was the purpose in this series of experiments to study whether or not a given quantity of the fixing substance can be detected, not only at the usual incubation temperature of 37° c., but also at 30° or 23°. Graduated doses of a strongly positive syphilitic serum were chosen, so that a series would represent two and one fixing units and three-fifths and one-fifth of a unit. For the first experiment a serum was selected which produced complete fixation in a dose of 0.05 c.c. within 30 minutes at 37° c. (water-bath). Of this 0.1, 0.05, 0.03, and 0.01 c.c. were measured into different tubes and tested for their fixing capacity at different temperatures, 37°, 30°, and 23° c.

In the first series human complement, 0.1 c.c.,

and in the second guinea-pig complement, 0.04 c.c. (or 0.1 c.c. of 40 per cent guinea-pig serum dilution), were used. The quantities of the other elements were as usual, antihuman amboceptor, 0.1 c.c., containing one unit for the human and two units for the guinea-pig complement, 10 per cent human corpuscle suspension, 0.1 c.c., and antigen, 0.1 c.c. The total volume in each tube was made 1.5 c.c.

Four groups of tubes, representing 0.1, 0.05, 0.03, and 0.01 c.c. of the serum, were prepared for studying the influence of temperature upon the velocity of the reaction (Table xv). Each group had seven duplicate sets for each dose of serum, as it was necessary to add amboceptor and corpuscle suspension at seven different intervals after the first incubation period began. The addition of the amboceptor and corpuscle suspension was made simultaneously 10 minutes, 20 minutes, 30 minutes, 60 minutes, 2 hours, and 4 hours after the contents were mixed.

For the temperature of 37° C. a water-bath was used, for 30° C. a special thermostat room, and for 23° C. the laboratory room. Not only the first incubation, but also the second or hemolytic phase of the reaction, was carried out at the temperatures indicated; that is, the results recorded under the heading of 23° C. were obtained at that temperature throughout, and those at 30° and 37° C. also.

In the experiments recorded in Tables xv and

TABLE XV

FIXATION OF HUMAN COMPLEMENT AT VARIOUS TEMPERATURES

Incubation period	37° C. Serum				30° C. Serum				23° C. Serum			
	0.1 c.c.	0.05 c.c.	0.03 c.c.	0.01 c.c.	0.1 c.c.	0.05 c.c.	0.03 c.c.	0.01 c.c.	0.1 c.c.	0.05 c.c.	0.03 c.c.	0.01 c.c.
Immediately	–	–	–	–	–	–	–	–	–	–	–	–
10 minutes	+++	++	–	–	+++	+	–	–	+++	–	–	–
20 minutes	+++	++	+	–	+++	++	+	–	+++	+	–	–
30 minutes	+++	+++	++	±	+++	++	++	±	+++	++	±	–
60 minutes		+++	++	±	+++	+++	++	±	+++	+++	+	–
2 hours						+++	++	±	+++	+++	++	±
4 hours							++	±	+++	+++	++	±

Human complement, 0.1 c.c.; diluted antihuman amboceptor, 0.1 c.c. (one unit); 10 per cent human corpuscle suspension, 0.1 c.c.; antigen, 0.1 c.c. Total volume, 1.5 c.c.

The second incubation also was carried out at the temperatures indicated.

xvi the same quantities of fixing substance ("syphilitic antibody") were employed, but the source and amount of complement used were different. It will be noted that the reaction was somewhat stronger when 0.04 c.c. of guinea-pig complement was used instead of 0.1 c.c. of human complement. The difference seems to be due to the fact that the more active the complement the easier it is fixed, since guinea-pig serum contains the requisite complement in a much smaller volume.

Some striking facts are brought out in these experiments. When the amount of fixing substance exceeds two units (0.1 c.c.), complete fixation takes place within 20 minutes at 23° or 30° C., while at 37° C. the fixation is complete within 10 minutes. When one unit of fixing substance (0.05 c.c.) is used, the fixation is complete within 30 minutes at 37° C., 60 minutes at 30° C., and 2 hours at 23° C. With three-fifths (0.03 c.c.) and one-fifth (0.01 c.c.) of the fixing unit the minimum time required for completion of the fixation is 30 minutes for 37° C., 60 minutes for 30° C., and 2 hours for 23° C.

The velocity of the reaction of complement fixation and subsequent hemolysis is much greater at 37° C. than at 30° or 23° C. A quantity of complement which will completely hemolyze the mixture in 20 minutes at 37° C. will require 35 minutes at 30° C. and 45 minutes at 23° C. for complete hemolysis.

TABLE XVI

FIXATION OF GUINEA-PIG COMPLEMENT AT VARIOUS TEMPERATURES

Incubation period	37° C. Serum				30° C. Serum				23° C. Serum			
	0.1 c.c.	0.05 c.c.	0.03 c.c.	0.01 c.c.	0.1 c.c.	0.05 c.c.	0.03 c.c.	0.01 c.c.	0.1 c.c.	0.05 c.c.	0.03 c.c.	0.01 c.c.
Immediately........	−	−	−	−	−	−	−	−	−	−	−	−
10 minutes........	+++	++	+	−	+++	++	−	−	+++	++	−	−
20 minutes........	+++	+++	++	−	+++	++	+	−	+++	++	±	−
30 minutes........	+++	+++	++	±	+++	+++	++	−	+++	++	+	−
60 minutes........	+++	++	++	±	+++	+++	++	±	+++	+++	++	−
2 hours...........					+++	+++	++	±	+++	++	+	±
4 hours...........					+++	+++	++	±	+++	++	+	±

Forty per cent guinea-pig complement, 0.1 c.c.; diluted antihuman amboceptor, 0.1 c.c. (representing two units for this system); 10 per cent human corpuscle suspension, 0.1 c.c.; antigen, 0.1 c.c. Total volume, 1.5 c.c.

The second incubation also was carried out at the temperatures indicated.

It is therefore understood that the test of syphilitic serum at 30° or 23° c. requires a period of nearly 3 hours, while at 37° c. the reaction is complete at the end of 1 hour.

Examination of Specimens of Serum and Cerebrospinal Fluid at Different Temperatures. With a view to the possible practical application of the facts just mentioned, a series of tests was undertaken with specimens of serum and cerebrospinal fluid at 37°, 30°, and 23° c. The serum was used in the active state, and the cerebrospinal fluids were tested by adding human complement, 0.1 c.c., to each specimen. Table XVII gives the results.

There was no disagreement in the results obtained at the three different temperatures. Apparently the examination of syphilitic sera and cerebrospinal fluids can be conducted in a warm laboratory without recourse to an incubator in which a temperature of 37° c. is maintained. It is advisable, however, to make use of the incubator whenever it is available, and, when not accessible, to make the room as warm as possible, in order to accelerate the reaction. One hour of incubation is sufficient when the temperature of the air is 32–37° c.

DISCUSSION

Examination of syphilitic serum or cerebrospinal fluid can be made at any temperature between 23°

TABLE XVII

COMPARATIVE STUDY OF SPECIMENS TESTED AT DIFFERENT TEMPERATURES

Specimen	Amount, c.c.	37° C. Incubation 30 min.	30° C. Incubation 2 hrs.	23° C. Incubation 2 hrs.
Serum 1................	0.2	—	—	—
" 2................	0.2	++++	++++	++++
" 3................	0.2	—	—	—
" 4................	0.2	—	—	—
" 5................	0.2	—	—	—
" 6................	0.2	++++	++++	++++
" 7................	0.2	++++	++++	++++
" 8................	0.2	—	—	—
" 9................	0.2	+++	+++	+++
" 10................	0.2	—	—	—
" 11................	0.2	++	++	++
" 12................	0.2	—	—	—
" 13................	0.2	++++	++++	++++
" 14................	0.2	+	+	+
" 15................	0.2	—	—	—
" 16................	0.2	—	—	—
" 17................	0.2	—	—	—
" 18................	0.2	±	±	±
" 19................	0.2	—	—	—
" 20................	0.2	—	—	—
Cerebrospinal fluid 1.....	0.5	++++	++++	++++
Cerebrospinal fluid 2.....	0.5	++++	++++	++++
Cerebrospinal fluid 3.....	0.5	—	—	—
Cerebrospinal fluid 4.....	0.5	—	—	—
Cerebrospinal fluid 5.....	0.5	+++	+++	+++

Human complement was employed. Diluted antihuman amboceptor, 0.1 c.c. (one unit); 10 per cent human corpuscle suspension, 0.1 c.c.; antigen, 0.1 c.c. Total volume, 1.5 c.c.

The first and second incubations were carried out at the temperatures indicated.

and 37° c. The velocity of the fixation reaction, including the fixation of complement and subsequent hemolysis, is greater at a higher temperature, the optimum point being 37° c. The maximum reaction is also reached, however, when the mixture of lipoids, syphilitic serum, and complement is allowed to stand for a long enough period at a lower temperature, the minimum thermal point being near 23° c. For the optimum temperature (37° c.) an incubation of 30 minutes is sufficient, while for the minimum temperature (23° c.) 2 hours are necessary. At the temperature of 30° c. the reaction proceeds with moderate velocity and is complete within 60 minutes.

Guinea-pig complement gave a sharper reaction with the sera which contained less than one unit of the fixing substance. Fixation is complete, however, at any of the three temperatures within 20 minutes when there are more than two units present. A serum containing one unit of fixing substance will complete reaction within 30 minutes at 37° c., 60 minutes at 30° c., and 2 hours at 23° c., irrespective of whether human or guinea-pig complement is used.

For many reasons a properly adjusted thermostat for 37° c. is recommended for conducting the serum diagnosis of syphilis when possible, but it should not be overlooked that at a temperature near 30° c. an entirely reliable result can be obtained without a

special incubator. Even at a temperature as low as 23° C. the test can be carried out if sufficient length of time is allowed.

The foregoing statements refer only to the systems in which the acetone-insoluble fraction of tissue lipoids is used as antigen.

That complete fixation takes place after a long period of incubation, even at 4° C., has frequently been noted by various observers, and in some laboratories it is the practice to carry out parallel series of tests, one at 37° C. and another in the ice box. It has been found that the percentage of positive reactions is higher when fixation is carried out at 4° C. In this instance the second part of the reaction (hemolytic phase) is of course carried out at 37° C. as hemolysis does not take place at such a low temperature.

Finally the writer wishes to emphasize once more that the procedures given in this chapter were chiefly developed for an emergency such as we were confronted with during the latter part of the World War of 1914–1918, and, while perfectly reliable for diagnostic work, are less suited for quantitative analysis of the antibody contents.

CHAPTER X

The Wassermann System

AS in any complement-fixation test five different factors are required: antigen, patient's serum, complement, amboceptor, and blood-corpuscles. The source and mode of preparation of these factors are given in detail below.

PREPARATION OF ANTIGEN

Aqueous extracts as given below are no longer employed as antigens and are described here only because of historical interest.

Wassermann's Method. The liver or spleen of a congenitally syphilitic fetus is preferable. Cut the organ into very small pieces with a pair of scissors and mix the tissue with four parts of physiological salt solution to which phenol in the proportion of 0.5 per cent is added. For example:

> 360.0 c.c. salt solution (0.85 per cent)
> 100.0 grams liver
> 40.0 c.c. phenol (5 per cent)

This mixture is thoroughly shaken in a dark bottle for twenty-four hours by means of a shaking machine. The tissue pieces are separated by centrifugation, and the brownish, opalescent supernatant fluid is used for antigen. It should be preserved in a rub-

ber-stoppered dark flask in the refrigerator. Upon standing a precipitate falls to the bottom of the container. As much of the clear supernatant fluid as is necessary for the day's work should be poured off and the remainder put back on ice immediately.

As to the stability of this extract, there is no agreement among investigators, whose experiences differ widely upon this question. Wassermann, Neisser, Bruck and Schucht found that it is very unstable, soon becoming too anticomplementary for use. Citron once prepared a watery solution of antigen which he divided into three portions. He kept one part for his own use and the other two he sent to other laboratories. From one of these he received a report after the lapse of a week that the antigen had become inactive; from the other he received a similar report after four weeks. The portion which he kept was unaltered three months after its preparation. Hence it would seem that the stability of the antigen depends greatly upon the mode of its preservation. According to the writer's experience, the liver of a congenitally syphilitic fetus does not necessarily yield a good antigen, and once prepared as described it may deteriorate within a few weeks.

A normal organ should be similarly prepared for use as control.

Marie and Levaditi's Method. Mash the liver of a congenitally syphilitic fetus, dry in a vacuum, and

then pulverize it. The powder is suspended in four parts of physiological salt solution and the mixture centrifuged after twenty-four hours' extraction. The clear supernatant fluid is used.

Morgenroth and Stertz's Method. Preserve the organ (syphilitic liver) in the frozen state, and cut off a small piece each time for use in the test. Mash this bit of tissue with sea-sand and extract it with four parts of physiological salt solution. Filter through paper, and use the filtrate.

The most important point concerning antigen is to employ the proper quantity in the test. It has been made a general rule that that dose of antigen must be selected which does not bind complement even when the antigen is used in double quantity. The usual aqueous preparation may be used in 0.1 c.c. or 0.2 c.c. doses.

ALCOHOLIC EXTRACTS

Alcoholic extracts soon replaced aqueous preparations as antigen because of their stability. The socalled syphilis antigen of to-day is an alcoholic extract of liver or heart muscles of man, ox or guinea-pig. The consensus of opinion is that syphilitic liver has no advantage over the extract of normal organs. As has already been stated these alcoholic extracts should be used with inactivated serum only and

should be carefully selected. The writer prefers his acetone-insoluble fraction to the crude extract even with inactivated serum, because a larger number of antigenic units may be used without risk of anti-complementary action.

Porges and Meier's Method. Cut up a normal or syphilitic liver into small pieces, extract with five volumes of absolute alcohol for twenty-four hours, and filter through coarse filter-paper. The filtrate is evaporated in a vacuum at a temperature below 40° c. The sticky mass resulting is then used to prepare a 1 per cent suspension in physiological salt solution with the addition of 0.5 per cent of phenol. This emulsion is well shaken and filtered through fine paper. The minimal dose which shows inhibition of hemolysis is determined by titration, and half of this amount is used for the test. With a strong syphilitic serum 0.025 c.c. may give complete fixation, but 0.2 or 0.3 c.c. is usually necessary. The authors considered a preparation of lecithin (Kahlbaum) to be equivalent in antigenic property to the alcoholic extract, but later investigators have found such a preparation unreliable as an antigen.

Landsteiner, Müller and Pötzl's Method. Extract mashed guinea-pig's heart or liver with alcohol for about ten to twelve hours at 60° c., in the ratio of one gram of tissue to 50 c.c. of 95 per cent alcohol. Filter through paper and preserve the filtrate at

room temperature. Use two drops of the solution for the test.

Michaelis and Lesser's Method.   Shake minced normal or syphilitic liver with ten volumes of absolute alcohol for ten to twelve hours. Use glass beads to facilitate thorough extraction. After twenty-four hours the clear supernatant portion is poured or pipetted off and used as antigen. At the time the test is made one part of this extract is mixed with four parts of physiological salt solution and 1 c.c. of the resulting emulsion used. The emulsion becomes milky and tends to form a precipitate on standing and should be thoroughly shaken before use. Later Michaelis advocated the use of an alcoholic extract of normal human heart which has since been recommended by some American and English workers.

Browning and McKenzie's Method.   Browning and McKenzie advocated the use of a lecithin-cholesterin mixture as antigen. Lecithin was known to possses excellent qualities as antigen in the Wassermann reaction, but this property has since been found to be inconstant with different preparations. Cholesterin, once thought by Fleischmann to be capable of serving as antigen, was found to be without such property and quite indifferent to complement when mixed with inactivated nonsyphilitic human serum, although it often causes the disappearance or diminution of complement activity if

mixed with unheated human serum. Browning and McKenzie found that when cholesterin is added to lecithin the antigenic value of the latter is improved. They recommended dissolving 0.4 grams of cholesterin in every 100 c.c. of 3 per cent lecithin solution in absolute alcohol. For use in the Wassermann reaction this cholesterinized lecithin solution is diluted with physiological salt solution in varying proportions. Usually an emulsion of 1:16 dilution is prepared, which is then tested for its anticomplementary property with normal serum and complement. One-third of the quantity that no longer interferes with the action of complement in the presence of normal human serum is recommended for the test.

Method of Sachs, and of McIntosh and Fildes. Sachs, and McIntosh and Fildes replaced the lecithin of the antigen just described with an alcoholic extract of human heart muscle. That the latter yields an excellent antigen without cholesterin had long been known, but by the addition of cholesterin its antigenic value is considerably enhanced. For example, a sample of alcoholic heart extract with an inferior antigenic titre may be rendered highly efficient through the addition of cholesterin. This increase in antigenic power is associated with a corresponding increase in anticomplementary activity, hence a possible false positive reaction with certain non-syphilitic sera must be guarded against. For this

reason it is extremely important to determine the quantity which, while giving the highest percentage of positive reactions with syphilitic sera, will not cause fixation with nonsyphilitic sera. This is done by using for the test one-fourth of that quantity which no longer interferes with the activity of complement in the presence of 0.2 c.c. of inactivated nonsyphilitic human serum.

The Author's Method. This method, in which the acetone-insoluble fraction of tissue lipoids is obtained by fractionation, has already been described in detail (p. 70). A similar fraction of tissue lipoids has been obtained by other methods (Bordet, Neymann and Gager, and the author, pp. 72–74). The author recommends the use of this type of antigen for all Wassermann reactions.

PATIENT'S SERUM

Blood is drawn from a vein and the serum which separates after clotting is inactivated, preferably within twenty-four hours after its withdrawal from the patient, at 56° c. for half an hour. Cerebrospinal fluid should be used without inactivation. The test doses are 0.1 c.c. and 0.2 c.c.

COMPLEMENT

One cubic centimeter of a 1:10 dilution in 0.85 per cent salt solution of fresh guinea-pig's serum is

used. If kept at a temperature near 0° to 5° C. it may be preserved as long as 48 hours. For methods of preserving complement see pp. 61–62.

AMBOCEPTOR

The amboceptor for the Wassermann system is produced by immunizing rabbits against sheep corpuscles. The writer has been successful in obtaining an amboceptor of high titre by using successive intraperitoneal injections of washed corpuscles in doses of 2, 4, 8, and 12 c.c. at intervals of four or five days, and bleeding the animal nine or ten days after the last injection. The corpuscles should be centrifuged at least twice with a large quantity of physiological salt solution and the original bulk of the defibrinated blood (which has been indicated by a mark on the centrifuge tube) restored by the addition of salt solution. The usual mode of immunization, however, consists in intravenous injections of several successive doses of 2 to 4 c.c., while Coca recommends daily intravenous injections of 1 c.c. for several days.

The unit of amboceptor should be determined by titrating against 1 c.c. of a 5 per cent suspension of washed sheep corpuscles, using 0.5 c.c. of 1:10 dilution of guinea-pig complement. Two units are used in the test.

CORPUSCLE SUSPENSION

One cubic centimeter of a 5 per cent suspension of washed sheep corpuscles is used. The blood should be fresh, not older than three days even when kept on ice.

TECHNIQUE OF THE TEST

For historical interest the original Wassermann method with an aqueous antigenic extract is described in Table XVIII. It is interesting to note the precautions necessary to insure a reliable reaction. The large number of controls required in using an aqueous extract were rendered unnecessary by the introduction of alcoholic extracts.

Put the required amounts of serum, complement, and antigen into the respective tubes, and bring the total quantity of the mixture up to 3 c.c. by the addition of salt solution. Mix the contents of the tubes well and incubate in the thermostat for one hour at 37° C. At the end of this period add to every tube amboceptor and corpuscle suspension in the quantity prescribed, mix well, and incubate again for two hours. Then remove the tubes to an ice chest for twenty hours, when the test is complete and the results are ready for reading.

If the test has been properly carried out, there will be complete hemolysis in every control tube excepting

in the tube containing syphilitic serum and syphilitic antigen (positive control, see Table xviii). If the control tubes are satisfactory, the tubes containing the sera to be examined can be read for the final result. In this series all the tubes not containing a syphilitic organ extract should be completely hemolyzed.

In the tubes containing the unknown serum and a syphilitic organ extract, there may or may not be hemolysis according as the serum contains syphilitic antibodies or not. In the former event there will be inhibition of hemolysis, either total or partial; in the latter, the tubes should be completely hemolyzed.

The degree of inhibition of hemolysis varies according to the amount of syphilitic antibody present; if this is large in amount, or, in other words, if complete inhibition occurs in the tube containing 0.1 c.c. serum and 0.1 c.c. antigen, the result can be graphically represented, according to Citron, as $++++$. If inhibition of hemolysis is incomplete in the tube containing 0.1 c.c. of serum but complete in that containing 0.2 c.c., the result is recorded as $+++$. These reactions are usually called strongly positive. If the tube containing 0.1 c.c. serum is completely hemolyzed while that containing 0.2 c.c. shows complete inhibition, the result is expressed as $++$. Incomplete inhibition in the tube containing 0.2 c.c. is regarded as $+$. The last two reactions are called

TABLE XVIII

THE WASSERMANN SYSTEM (CITRON) WHEN AQUEOUS ORGAN-EXTRACTS ARE USED AS ANTIGEN

	Syphilitic liver, aqueous extract	Normal liver, aqueous extract	Guinea-pig's complement 1:10 dilution	Five per cent suspension of washed sheep corpuscles	Immune antisheep amboceptor (litre 1:1000) 1:1000 dilution	Results should be
Patient's serum (56° C.)						
0.2 c.c.	0.2 c.c.	. . . . .	1 c.c.	1 c.c.	1 c.c.	The degree of hemolysis in these 2 tubes determines the nature of this serum.
0.1 c.c.	0.1 c.c.	. . . . .	1 c.c.	1 c.c.	1 c.c.	
0.2 c.c.	. . . . .	0.2 c.c.	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis.
0.1 c.c.	. . . . .	0.1 c.c.	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis.
0.4 c.c.	. . . . .	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis.
0.6 c.c.	. . . . .	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis.
Positive syphil. serum (56° C.)						
0.2 c.c.	0.2 c.c.	. . . . .	1 c.c.	1 c.c.	1 c.c.	No hemolysis
0.1 c.c.	0.1 c.c.	. . . . .	1 c.c.	1 c.c.	1 c.c.	No hemolysis
0.2 c.c.	. . . . .	0.2 c.c.	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
0.1 c.c.	. . . . .	0.1 c.c.	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
0.4 c.c.	. . . . .	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
0.6 c.c.	. . . . .	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
Normal serum (56° C.)						
0.2 c.c.	0.2 c.c.	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
0.1 c.c.	0.1 c.c.	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
0.2 c.c.	. . . . .	0.2 c.c.	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
0.1 c.c.	. . . . .	0.1 c.c.	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
0.4 c.c.	. . . . .	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
0.6 c.c.	. . . . .	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
No serum	0.4 c.c.	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
No serum	0.6 c.c.	. . . . .	1 c.c.	1 c.c.	1 c.c.	Complete hemolysis
No serum	. . . . .	0.4 c.c.	1 c.c.	1 c.c.	1 e.c.	Complete hemolysis
No serum	. . . . .	0.6 c.c.	1 c.c.	1 c.c.	1 e.c.	Complete hemolysis
No serum	. . . . .	. . . . .	1 c.c.	1 c.c.	. . . . .	No hemolysis
No serum	. . . . .	. . . . .	1 c.c.	1 c.c.	1 c.c.	No hemolysis

Incubation at 37° C. for 1 hour.

The volume of each tube is now brought up to 3 c.c. with salt solution.

The volume of each tube is now 3 c.c. Mix the contents of tubes thoroughly and incubate at 37° C. for 2 hours. Remove tubes then to refrigerator over night and read the reactions after this period.

weakly positive. When inhibition in the tube con-
taining 0.2 c.c. is very slight the result is recorded
as ±.

The original method of Wassermann has been
essentially modified by the substitution, for aqueous
antigens, of alcoholic extracts of hearts or acetone-
insoluble tissue lipoids, the advantage of the alco-
holic antigen preparations being that they can be
preserved for some time without developing anti-
complementary properties. Titration is of course
necessary, according to the requirements already
indicated (pp. 74–78). A uniform dosage of antigen is
used (1 c.c. of a 1:10 dilution). The amounts of
serum are 0.1 and 0.2 c.c., in order that the intensity
of the reaction can be roughly estimated. The total
volume has been reduced from the original 5 c.c. of
Wassermann to 2.5 or less, with corresponding
reduction in all of the reagents except the syphilitic
serum and antigen.

The regular Wassermann method has been greatly
simplified by the use of acetone-insoluble tissue
lipoids as antigen. Other investigators, as well as
the writer, have found that there is no essential dif-
ference between the antigen extract thus prepared
and the aqueous extract, while the former in the
hands of the writer has proved much more stable
and does away with the necessity of using the normal
organ-extract controls which rendered the original

TABLE XIX

THE ANTISHEEP HEMOLYTIC SYSTEM, WHEN ALCOHOLIC TISSUE EXTRACTS OR ACETONE-INSOLUBLE TISSUE LIPOIDS ARE USED AS ANTIGEN

	Standard antigen emulsion	Guinea-pig complement, 1:10 dilution		Five per cent suspension of the washed sheep's corpuscles	Antisheep immune amboceptor (titre 1:2000) 1:1000 dilution		Results should be
Patient's serum (56° C.)							
0.1 c.c.	0.1 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.		These two tubes show variable reactions according to the nature of the serum
0.2 c.c.	0.1 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.		
0.2 c.c.	....	0.5 c.c.		0.5 c.c.	0.5 c.c.		Complete hemolysis
Positive syphil. serum (56° C.)							
0.1 c.c.	0.1 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.		No hemolysis (positive reaction)
0.1 c.c.	....	0.5 c.c.		0.5 c.c.	0.5 c.c.		Complete hemolysis
Normal serum (56° C.)							
0.1 c.c.	0.1 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.		Complete hemolysis (negative reaction)
0.1 c.c.	....	0.5 c.c.		0.5 c.c.	0.5 c.c.		Complete hemolysis
	0.2 c.c.	0.5 c.c.		0.5 c.c.	0.5 c.c.		Complete hemolysis (should be no inhibition)
0.1 c.c.	....	0.5 c.c.		0.5 c.c.	0.5 c.c.		Complete hemolysis (should be no inhibition)
	....	0.5 c.c.		0.5 c.c.	0.5 c.c.		Complete hemolysis

(Column between complement and corpuscles): Make the volume of each tube 1.5 c.c. Mix the contents well and incubate at 37° C. for one hour (thirty minutes in a water thermostat).

(Column between amboceptor and results): The total volume is 2.5 c.c. each. Mix the contents well and incubate at 37° C. for two hours (one hour in a water thermostat). Reading of the results after four to six hours at room temperature.

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Wassermann method so complicated and cumbersome.

In Table xix the exact method of performing the test is given. It will be noted that all the reagents have been used in half the quantity of the original Wassermann method. A uniform dosage of antigen is used, which, in the experience of the writer, has been sufficient to show the varying intensity of the reaction.

CHAPTER XI

Diagnostic Value of the Serum Reaction of Syphilis

THE phenomenon of complement-fixation in syphilis is a type of reaction distinct in itself, differing widely from all other known examples of complement-fixation based on the Bordet-Gengou principle. The chief difference between the two types of phenomena arises from the nonspecific nature of the substances that function as antigen in the Wassermann reaction. In the Bordet-Gengou phenomenon, as we have already seen (p. 23), the complement is absorbed or fixed only when brought into contact with combinations of specific antigens and antibodies. In general it may be said that the specificity of these antigens and antibodies can be compared in a way to the relation which exists between locks and keys, and it can be stated that they do not interact with one another unless they are in exact correspondence. On the other hand the phenomenon of Wassermann is produced by the interaction of substances in the serum (once regarded as specific antibody) and certain tissue lipoids, both of which are now known to be nonspecific. Therefore in this case the law of specificity does not operate in the same strict sense as in other known examples of the Bordet-Gengou phenomenon.

TABLE XX
BLOOD-SERUM

Investigators	Primary syphilis		Secondary syphilis manifest		Tertiary syphilis manifest		Early latent syphilis		Late latent syphilis	
	No. of cases	Per cent +	No. of cases	Per cent +	No. of cases	Per cent +	No. of cases	Per cent +	No. of cases	Per cent +
Wassermann, Neisser, Bruck and Schucht.............	25	91	101	73.3	37	78.4	41	85.4	53	88.7
Citron and Blaschko........	64	90	56	98	23	91	67	80	51	57
Bruck and Stern............	27	48.2	163	79.1	47	57.4	50	20	79	20
Bruhns and Halberstädter..	9	88.9	50	98	16	100	39	43.3	82	28
Ledermann..................	19	52.6	56	100	27	92	41	75.6	19	36.8
Ledermann..................	46	61.2	110	98.1	78	96.2	115	83.8	78	53.8
Lesser.....................	56	69	204	91	131	90	118	67	425	46
Noguchi**..................	33	66.6	120	86.6*	91	72.5	81	48.1	74	44.7
Hoehne.....................	44	38.6	329	79.1	33	63.6	387†	31.3		
Boas.......................	50	60	395	100	63	97	294†	47		
Detre and Brezovsky........	43	98	21	81	35	73				
	416	69.8	1605	89.4	581	78.1	1233	51	861	47

* Exclusive of treated cases 98 per cent positive results were obtained.
† No distinction was made between the late and early latent cases.
** Not including 1800 cases examined by the author's antihuman hemolytic system.

TABLE XXI
BLOOD-SERUM

Investigators	Hereditary syphilis		Cerebrospinal syphilis		General paralysis		Tabes	
	No. of cases	Per cent +	No. of cases	Per cent +	No. of cases	Per cent +	No. of cases	Per cent +
Boas and Thomsen	32	87.5						
Bauer	22	100						
Halberstädter, Müller, and Reiche	27	92						
Ledermann	16	100	26	88.5	23	87	68	76.4
Hoehne	24	87.5	12	16.7	30	80	45	60
Noguchi*	4	100	2	50			22	40.9
Boas					42	100	20	80
Nonne			?	(20)	?	(90)	?	(90)
Lesser					62	100	61	56
Frenkel-Heiden			7	27	14	78.5		
Plaut			4	25	180	100		
Citron and Blaschko			10	60				
Stertz			3	66	45	95.5		
Marie, Levaditi, and Yamanouchi					30	59		
Raviart, Breton, Petit					72	93		
	125	94.5	64	47.6	498	88.1	216	62.66

* Not including 362 cases of tabes, 192 cases of general paralysis, 48 cases of hereditary lues, 11 cases of cerebrospinal lues examined by the author's antihuman system.

Notwithstanding its nonspecific character the presence of the Wassermann reaction has proved to be one of the most constant signs of syphilitic infection and nearly always indicates the existence of the disease. There was a period when the value of the Wassermann reaction was much debated, but today no one insists on its infallibility as a diagnostic method or blindly rests his diagnosis on the strength of a single Wassermann test. The early statistical data furnish, therefore, all that is expected of this reaction in different stages of syphilitic infection (see Tables xx and xxi).

In primary syphilis the results vary much, ranging from 98 per cent (Detre) to 38 per cent (Hoehne). This difference may be accounted for, aside from technical considerations, by the state of the infection at the time of examination. Early cases of chancre frequently give a negative reaction. However, in this stage of syphilis the test is not usually necessary excepting in those cases in which a differentiation between chancroidal and mixed infection is desirable, and in cases of suspected intra-urethral chancre.

In the following table are presented the data collected by Craig concerning the time of appearance of the positive reaction in primary cases. The earliest was five days after the initial lesion had appeared.

TABLE XXII

DATE OF APPEARANCE OF POSITIVE REACTION IN 31 CASES OF LUES

Days after initial lesion	No. of cases positive
5	1
8	2
11	2
13	3
14	1
17	2
18	2
19	2
20	1
21	2
22	1
23	1
25	2
28	1
29	2
30	6

Craig later (1920 edition, The Wassermann Test) obtained, in a series of 600 cases, positive Wassermann reactions in 36 per cent at the end of the first week after the appearance of the chancre, in 60 per cent at the end of the second, 70 per cent at the end of the third, 77 per cent at the end of the fourth, and 80 per cent at the end of the fifth week. Klauder obtained positive reactions within the first 10 days after appearance of chancre in 36 per cent of his cases, within 20 days in 64 per cent, within 30 days in 70 per cent, and within 40 days in 100 per cent. Comparative studies by Vedder and Klauder showed that during the early period both Wassermann reaction

and darkfield examination may fail to demonstrate
the presence of the infection, but the demonstration
of the organism is the first positive finding (60 to 90
per cent of Klauder's cases), while after the fifth week
the organism is found only in 30 per cent of cases.

In secondary syphilis the highest figures are those
of Boas and of Ledermann; the former obtained 100
per cent of positive reactions in 395 cases, the latter
a similar result in 56 cases. The lowest figures are
recorded by Hoehne, who obtained 79.1 per cent of
positive reactions in 329 cases, and by Bruck and
Stern, who examined 163 cases with similar results.
The variations in these figures cannot well be ac-
counted for unless an analysis of the stage of the
disease and the treatment received by the patient at
the time of test are taken into consideration. The
reaction in this stage of syphilis is fairly constant
and is a reliable index of the presence of syphilitic
"antibodies" in the patient's serum.

In tertiary lues the figures vary from 57.4 per cent
(Bruck and Stern in 47 cases) to 100 per cent (Bruhns
and Halberstädter in 16 cases). Here again the
effect of treatment is not taken into account.

In early latent cases the figures vary from 20 per
cent (Bruck and Stern in 50 cases) to 85 per cent
(Wassermann, Neisser, et al. in 41 cases). By early
latent cases are meant late secondary cases with-
out symptoms. In late latent cases, or those follow-

ing the manifest tertiary stage, without symptoms, the figures show about the same results. The technique of the various investigators and the reagents used by them must be taken into account in interpreting their results.

General paralysis shows fairly constant positive reactions, ranging from 80 per cent to 100 per cent of cases examined. Tabes gives a somewhat lower percentage, from 40 per cent to 80 per cent. In hereditary syphilis the figures are rather constantly high, the lowest being 87.5 per cent. In cerebrospinal lues the results vary from 16 per cent in 12 cases reported by Hoehne to 88.5 per cent in 26 cases reported by Ledermann.

For comparison there are inserted the results obtained by different investigators with the antihuman hemolytic system in 3580 cases of syphilis in its various manifestations and stages. Out of this total number 1771 cases were examined by the Wassermann system at the same time. The results of comparison show clearly that the former gives a higher percentage of positive reactions than the Wassermann (Tables XXIII, XXIV and XXV).

Orleman-Robinson made an extensive study expressly for the purpose of determining whether or not the use of active serum in the writer's system would give an occasional positive reaction in nonsyphilitic dermatological conditions. Her results with

TABLE XXIII

PERCENTAGE OF POSITIVE REACTIONS OBTAINED BY THE AUTHOR'S ANTIHUMAN (N.) SYSTEM AS COMPARED WITH WASSERMANN'S (W.)

	Primary syphilis			Secondary syphilis			Tertiary syphilis			Latent syphilis			Congenital syphilis			Cerebrospinal syphilis			Total
	No. of cases	W. %	N. %	No. of cases	W. %	N. %	No. of cases	W. %	N. %	No. of cases	W. %	N. %	No. of cases	W. %	N. %	No. of cases	W. %	N. %	
Noguchi............	23	73.9	86.9	79	83.7	96.2	65	80.0	87.6	59	61.0	75.5	4	100.0	100.0	5	....	80.0	235
Fox...............	7	100.0	100.0	37	97.0	100.0	32	71.0	84.0	54	46.0	62.0	1	100.0	100.0	..	....	....	131
Kaplan............	138	90.0	97.0	281	86.0	98.0	191	73.0	81.0	79	51.0	75.0	20	90.0	90.0	..	....	....	709
Swift.............	16	81.0	81.0	76	92.0	97.0	45	80.0	88.0	85	55.0	62.0	4	100.0	100.0	..	....	....	226
Corson-White......	14	86.0	100.0	146	98.0	99.0	47	80.0	80.0	28	60.0	64.0	39	100.0	100.0	35	80.0	80.0	309
Kaliski...........	10	100.0	100.0	50*	94.0	100.0	75†	60.0	80.0	..	....	....	11	100.0	100.0	15	66.0	80.0	161
Total..........	208	88.0	94.0	669	92.0	98.0	455	74.0	83.0	305	54.0	68.0	79	98.0	98.0	55	73.0	80.0	1777
ANTIHUMAN HEMOLYTIC SYSTEM ALONE																			
Noguchi...........	70	....	92.8	197	....	96.0	177	....	88.9	270	....	74.4	17	....	100.0	5	....	100.0	736
Craig.............	90	....	72.0‡	163	....	88.0	74	....	82.0	55	....	72.0	9	....	88.8	..	....	....	491
Orleman-Robinson..	29	....	86.0	48	....	93.0	60	....	80.0	33	....	69.6	10	....	100.0	..	....	....	180
Potter	7	....	86.0	71	....	98.6	46	....	78.0	58	....	66.0	..	....	....	..	....	....	182
Groat.............	12	....	100.0	76	....	94.4	36	....	70.0	57	....	40.0§	10	....	100.0	1	....	100.0	186
Berghausen........	..	....	....	15	....	93.0	9	....	88.0	6	....	66.0	4	....	75.0	..	....	....	34
Total..........	208	....	87.5	570	....	94.7	402	....	82.8	474	....	64.6	50	....	93.0	6	....	100.0	1809
Total, both systems..	416	88.0	90.0	1239	92.0	96.0	857	74.0	83.0	779	54.0	66.3	129	98.0	96.0	61	73.0	90.0	3580

* Untreated.   † Includes latent cases.   ‡ Includes very early cases.   § Majority under treatment.

TABLE XXIV

| | General paralysis | | | | | |
| | Blood-serum | | | Cerebrospinal fluid | | |
	Num-ber of cases	W.* per cent	N.† per cent	Num-ber of cases	W.* per cent	N.† per cent
Noguchi......................	25		86			
Rosanoff and Wiseman......	56		80	44		86
Corson-White..............	11	80	80	5	100	100
Kaplan.....................	61	65	72			
Kaliski.....................	3	66	66			
Schradieck.................	4		100			
Groat......................	2		100			
Total..................	162	70	73.4	49	100	93

* W. = Wassermann antisheep system.
† N. = Noguchi antihuman system.

TABLE XXV

| | Tabes | | |
| | Blood-serum | | |
	Number of cases	W.* per cent	N.† per cent
Noguchi.............................	125		68
Noguchi.............................	8	44	72
Kaplan.............................	205	60	65
Corson-White.......................	49	70	75
Kaliski.............................	10	40	60
Berghausen.........................	6		66
Fox.................................	3	100	100
Waugh.............................	13		56
Total.............................	419	62.8	72

* W. = Wassermann antisheep system.
† N. = Noguchi antihuman system.

236 cases were uniformly negative (Table XXVI).
For controls 63 cases of syphilis were also examined,
results of which are quoted elsewhere (pp. 203–204).

TABLE XXVI

Acne vulgaris	18	Ichthyosis	4
Acne rosacea	6	Impetigo contagiosa	14
Alopecia areata	10	Lichen planus	10
Dermatitis herpetiformis	3	Lichen rubra pilaris	1
Dermatitis traumatica	6	Lupus erythematosus	10
Dermatitis venenata	4	Lupus vulgaris	4
Eczema	25	Molluscum contagiosum	3
Epithelioma	30	Nævus pigmentosus	3
Erythema multiforme	5	Pityriasis rosea	4
Erysipelas	3	Pityriasis versicolor	3
Erysipeloid	3	Psoriasis	15
Favus	2	Purpura	5
Scabies	6	Trichophytosis	10
Herpes zoster	10	Tuberculosis cutis	3
Hydrocystoma	3	Urticaria	13

TABLE XXVII

CEREBROSPINAL FLUIDS

	General paralysis		Tabes		Cerebrospinal syphilis	
	No. of cases	Per cent +	No. of cases	Per cent +	No. of cases	Per cent +
Marie and Levaditi	39	73	9	66.6		
Marie, Levaditi, and Yamanouchi	30	93				
Stertz	45	88.8	5	60	8	0
Noguchi and Moore	60	73	11	54.5	6	50
Wassermann and Plaut	41	88				
Morgenroth and Stertz	8	100				
Plaut	54	90			4	0
Nonne	?	90	?	50	16	25
Schütze			12	66.6		
Marinesco	35	94	15	53		
Smith and Candler	64	92.1				
Noguchi, Rosanoff, and Wiseman	56	87.5				
	432	90	52	56.2	34	19

The results of the analysis of the cerebrospinal fluid in general paralysis vary from 73 per cent (Marie and Levaditi, and Noguchi and Moore) to 100 per cent (Morgenroth and Stertz). The results are uniformly high, especially when contrasted with tabes and cerebrospinal lues (Table xxvii). In tabes the figures vary from 54.5 per cent to 66.6 per cent. In cerebral syphilis the presence of the binding substance is very uncertain; Plaut examined four cases with uniformly negative results, while Henderson obtained positive reactions in most of his cases.

TABLE XXVIII

EXAMINATIONS OF BLOOD-SERUM AND CEREBROSPINAL FLUID IN CASES OF LEPROSY

	Serum		Spinal fluid	
	No. of cases	Per cent +	No. of cases	Per cent +
Eitner...................	2	100		
Wechselmann and Meier.....	1	100		
Slatineanu and Danielopolu..	26	100*	19	72
Slatineanu and Danielopolu..	21	57*	20	0
Jundell, Almqvist, and Sandman....................	26	30*		
Bruck and Gessner.........	10	50†		
Noguchi...................	10	70		
Fox.......................	60	53		
	146	61	39	36

* Including weak reactions.   † Five out of seven cases of tubercular form.

Certain investigators have reported a high percentage of positive reactions in leprosy (Table xxviii)

and in other nonspecific diseases, notably scarlet
fever (Table XXIX), carcinoma, and diabetes mellitus.[1]

TABLE XXIX
CASES OF SCARLATINA

	No. of cases	Per cent +		No. of cases	Per cent +
Much and Eichelberg	130	46	Seligmann and Klopstok.........	30**	
Jochmann and Töpfer	33	o	Boas and Hauge...	61	1.5†
Halberstädter, Müller, and Reiche....	10	50*	Bruck and Cohn...	28	o
			Noguchi...........	63	1.5†
Meier.............	52	1.8†	Fùa and Koch.....	57	25§
Hoehne............	37	2.5†	Hecht.............	106	1

* Weak reactions only, which became negative when tested with several
other extracts.

† One case showed some inhibition. The case reported by the writer was
subsequently proven to be a child with congenital lues.

** All negative in 13 cases examined on July 1–3, but 16 additional cases
examined one month later with the same antigen gave 3 weak and 13
strongly positive reactions. These investigators are inclined to think that
their antigen altered on standing, hence the positive results.

§ Weak reactions only, which finally disappeared on standing.

[1] A few investigators obtained positive reactions in an astonishingly
large proportion of nonsyphilitic cases, but the majority of the workers
do not get such results. Among those who reported a large number
of positive reactions in nonsyphilitic cases may be mentioned Weil and
Braun, Elias, Neubauer, Porges, and Salomon. Weil and Braun encountered 4 positive in 12 cases of pneumonia, 3 positive in 20 cases of typhoid
fever, 2 positive in 21 cases of tuberculosis, 1 positive in 4 cases of diabetes mellitus, and 2 positive in 11 cases of tumors. Elias and others
found 5 positive in 33 cases of tuberculosis and 4 positive in 14 cases of
tumors. Hancken met with 2 positive reactions in 28 control cases, one
being a subject with scarlatina and one other with diphtheria. Löhlein
examined 250 cases and obtained positive results in 4 cases of tuberculosis and carcinoma. Later investigators, especially those who had been
working with the reaction constantly, all failed to get the results just
described except for an occasional weakly positive reaction in cases of
carcinoma, scarlet fever, or diabetes. It is reasonable to suspect anyone
who obtains a high percentage of positive reactions in nonsyphilitic cases
of not doing the test properly.

In Table xxx is shown the writer's study of the results of 322 cases in which syphilis did not play an etiological part. In 8 cases of pellagra Bass reported positive reactions with the Wassermann system, but later investigations with the writer's system by Fox on 30 cases and by Litterar on 20 cases of the same disease gave negative results.

TABLE XXX

ANTIHUMAN HEMOLYTIC SYSTEM. CASES IN WHICH SYPHILIS CAN BE
EXCLUDED WITH A FAIR DEGREE OF CERTAINTY

	Cases examined	+	−	±
Carcinoma..................	51	1	50	0
Sarcoma...................	3	0	3	0
Adenosarcoma..............	1	0	1	0
Endothelioma..............	1	1	0	0
Scarlatina..................	62	0	60	2
Varicella...................	1	0	1	0
Measles....................	2	0	2	0
Tuberculosis................	52	0	52	0
Lupus:.....................	2	0	2	0
Banti's disease.............	1	1	0	0
Hodgkin's disease.	2	0	2	0
Muscular distrophy..........	5	0	5	0
Neurasthenia...............	2	0	2	0
Dementia præcox.......	5	0	5	0
Various skin diseases........	58	0	58	0
Miscellaneous...............	74	0	74	0
	322	3	317	2

In Tables xxxi and xxxii are given the results of examination of 132 cases of diseases in which syphilis may be an etiological factor and of 130 cases of eye

diseases of all sorts studied by Cohen and Bronfen-
brenner.

In certain nervous diseases of unknown origin the
Wassermann reaction has been resorted to as a means
of determining, if possible, the nature of the causa-

TABLE XXXI

ANTIHUMAN HEMOLYTIC SYSTEM, CASES IN WHICH SYPHILIS IS AN ETIOLOGICAL
FACTOR OR CANNOT BE EXCLUDED AS A POSSIBLE CAUSE OF THE CONDITION

	Cases examined	+	−	±
Cirrhosis of liver.............	7	5	1	1
Samples of ascitic fluid	21	11	9	0
Aortic insufficiency..........	1	1	0	0
Chronic arthritis.............	10	2	6	2
Eye cases...................	29	14	15	0
Diabetes...................	5	1	4	0
Eczema....................	32	1*	31	0
Scleroderma................	4	1	3	0
Brain tumor (?).............	8	4	4	0
Central gliosis (?)...........	2	1	1	0
Hemiplegia.................	8	3	5	0
Spastic paraplegia...........	3	2	0	1
Raynaud's disease†..........	2	0	2	0
	132	46	81	4

* This case has been reported also by Fox, in Table 8. For the other 31
cases I am indebted to Dr. Daisy Orleman-Robinson.

† Kaliski and Buerger, using Wassermann's and the writer's systems, got
negative results in 16 cases of thrombo-angiitis obliterans.

tive factor. For example, Raviart, Breton and Petit
examined patients suffering from various forms of
insanity, other than parasyphilitic affections, for
the presence of this reaction in the blood. Their

TABLE XXXII

ANTIHUMAN HEMOLYTIC SYSTEM

	Total number of cases	Undoubtedly syphilitic +	Undoubtedly syphilitic −	Doubtfully syphilitic +	Doubtfully syphilitic −	Under recent treatment +	Under recent treatment −
Interstitial keratitis............	38	7	2	17	12	5	7
Iritis........................	16	3	2	5	6	1	3
Iridocyclitis..................	3	0	1	0	2	0	1
Optic neuritis.................	10	2	0	4	4	2	0
Optic atrophy.................	10	0	1	2	7	1	0
Neuroretinitis................	6	0	2	3	1	0	1
Retrobulbar neuritis...........	1	0	0	0	1	0	0
Retinitis pigmentosa...........	8	0	1	5	2	0	1
Retinitis.....................	1	0	0	0	1	0	1
Detachment of retina..........	2	0	1	1	0	0	0
Embolism of central artery......	2	0	1	0	1	0	1
Chorioiditis..................	8	0	0	2	6	0	0
Chorioretinitis...............	6	0	0	3	3	3	1
Scleritis.....................	2	0	0	1	1	0	1
Ophthalmoplegia interna.......	2	1	1	0	0	0	0
Oculomotor paralysis..........	2	0	2	0	0	2	0
Ptosis.......................	2	0	0	0	2	0	0
Paralysis external rectus.......	2	0	0	0	2	0	0
Diplopia.....................	2	0	1	1	0	0	0
Corneal ulcer.................	1	1	0	0	0	0	0
Chancre of upper lid..........	1	0	0	1	0	0	0
Sympathetic ophthalmia........	1	0	0	0	1	0	1
Acromegaly..................	2	0	0	1	1	0	0
Amaurotic family idiocy........	1	0	0	0	1	0	0
Graves' disease...............	1	0	0	0	1	0	0
	130	14	15*	46	55	14	18

* Under antisyphilitic treatment.

results are somewhat striking; they got positive reactions in about 30 to 40 per cent of cases of epilepsy, idiocy, and imbecility, in 3 of 5 cases of dementia senilis and in 5 out of 19 cases of dementia præcox. Raubinovitch and Levaditi examined sera from 15 cases of dementia præcox and got positive

TABLE XXXIII

PSYCHIATRIC CASES

Clinical diagnosis	Blood-serum				Cerebrospinal fluid				Syphilis ascertained
	No. of cases	Reactions			No. of cases	Reactions			
		−	+	±		−	+	±	
Arteriosclerotic dementia..	10	10	0	0	9	9	0	0	
Brain tumor..............	1	1	0	0	1	1	0	0	
Traumatic psychosis.......	1	1	0	0	1	1	0	0	
Senile dementia...........	16	13	1	2	10	8	1	1	
Infantile cerebral palsy....	6	6	0	0	5	5	0	0	
Epilepsy.................	69	48	12	9	55	50	3	2	
Huntington's chorea.......	2	1	1	0	1	1	0	0	
Uremic psychosis.........	1	1	0	0	1	1	0	0	
Alcoholic psychosis........	9	4	2	3	6	4	1	1	4
Polyneuritic psychosis.....	8	7	1	0	8	8	0	0	
Involution melancholia....	10	8	2	0	7	7	0	0	
Dementia præcox.........	131	99	15	17	83	76	3	4	5
Manic depressive insanity.	14	9	2	3	7	5	2	0	1
Paranoiac condition.......	9	7	1	1	4	4	0	0	
Imbecility................	6	4	2	0	6	6	0	0	
Constitutional inferiority..	1	1	0	0	1	1	0	0	
Unclassified............	40	28	6	6	38	35	2	1	5
	334	248	45*	41*	243	222	12*	9*	15

* These cases showing positive and doubtful reactions may have had syphilis, but it was difficult to ascertain the disease in all the cases. In 15 cases at least, syphilis was proven to be present.

results in 20 per cent, but all the spinal fluids examined gave negative results. This last fact conforms with the observations of Moore and the writer. Rosanoff, Wiseman, and the writer examined 413 cases of various forms of insanity for the reaction in serum and cerebrospinal fluid and obtained results similar to those of previous investigators.

Atwood and Bronfenbrenner, using the antihuman system, examined the sera of 204 idiots and found 14.7 per cent positive reactions among them.

A rough classification of the cases is shown in the following list:

TABLE XXXIV

CLASSIFICATION OF THE 204 LOW-GRADE IDIOTS TESTED

Idiopathic	120
Diplegic	47
Hemiplegic	7
Epileptics without paralysis	13
Hydrocephalic	5
Microcephalic	6
Cretins	2
Myxedematous	1
Amaurotic family idiocy	1
Idiocy with cerebellar ataxia	2

TABLE XXXV

CLASSIFICATION OF THE 30 IDIOTS WHO SHOWED A POSITIVE WASSERMANN REACTION

	POSITIVE	TOTAL	PERCENTAGE
Idiopathic	13	120	10
Diplegic	11	47	23
Hemiplegic	2	7	28
Microcephalic	1	5	20
Epileptic without paralysis	1	12	8
Cerebellar ataxic	2	2	100

Four of the patients were blind, and four mute. There were other physical disorders, not syphilitic, in other cases. One of the diplegics with positive reaction was epileptic, one hydrocephalic, and one epileptic with mutism. One of the cerebellar ataxic patients was microcephalic, and one of the diplegics was blind. One out of four deaf-mutes showed a positive sero-reaction. The myxedematous idiot, the two cretins, and the patient with amaurotic family idiocy gave negative reactions. The percentage of positive reactions found was much greater, in proportion, in idiots with superadded gross organic brain defect than in idiopathic idiocy. These results are in close agreement with those of Lippmann, who employed the Wassermann system in Germany.

In gynecological conditions the reaction has also been called upon to test the validity of the laws of Colles and Profeta. Müller found that with the blood of wives of syphilitic husbands who had had repeated abortions and premature births the results were usually negative; the same was true of the blood of their offspring. Knöpfelmacher and Lehndorffer examined 32 apparently healthy mothers of syphilitic children and obtained positive reactions in 18. Halberstädter, Müller and Reiche found that the reaction may be negative with children of syphilitic mothers, and *vice versa*, while Boas and Thomsen assert that the reaction may develop later in children

whose blood gives a negative result at the time of birth. They all agree that the negative reaction in these children or mothers is largely due to the latency of the disease, but is not a sign of immunity against the disease. Thus, while the mother of a syphilitic infant may present no sign of syphilis, examination of the blood of the mother gives positive reaction in half the number of cases examined.

CHAPTER XII

Effect of Treatment upon the Reaction

MERCURIAL TREATMENT

MUCH work has been done by numerous investigators to determine the result of various forms of treatment upon the Wassermann reaction in the blood and cerebrospinal fluid. It is known that the reaction frequently disappears after a short course of treatment, often to return again within a longer or shorter period. Nichols found that syphilitic orchitis of rabbits is not perceptibly influenced by the administration of mercurial preparations even when given intravenously in large quantities.

Citron, who was among the first to investigate the effect of treatment upon the reaction, found that whereas before treatment the percentage of positive results obtained by him was 81, treatment had the effect of reducing the figures to 65 per cent. In about half of these cases, numbering 57 in all, only one course of treatment was given. Bruck and Stern obtained positive reactions in 81.5 per cent of 173 untreated cases, and in another group of treated cases got positive reactions in only 28 per cent. Blaschko studied 52 positive cases of manifest syphilis after treatment and found that 45 of them gave negative results; of 38 cases of latent syphilis 31 gave negative results after treatment. Hoehne studied 211 cases

which before treatment gave positive reactions, and found that in 56 per cent the reaction disappeared after therapeutic interference; 33.9 per cent gave a positive reaction notwithstanding some treatment. In 5 cases, after eleven to twelve injections of mercuric salicylate over a period of two months, the reaction was positive. Lesser states that a positive reaction can be made negative in about 35 per cent of cases by giving 30 inunctions of mercury, 12 injections of an insoluble mercuric preparation, or 25 injections of a soluble mercuric preparation. The rapidity with which the reaction disappears is very variable in different individuals. Boas found that after a course of injections during two or three months the reaction became negative in 76 out of 82 cases, and states that the reaction may return within a month after cessation of treatment, indicating a recurrence. V. C. Pedersen published an extensive classification showing the relation of the Wassermann reaction to the clinical course of the disease and to treatment. Craig arrived at the conclusion that the disappearance or reduction of the reaction during the treatment is a valuable indicator for the effectiveness of the treatment. Irregular and inadequate therapeutic measures, no matter how long they may be extended, leave the reaction still positive.

It is evident, then, that the reaction is usually

affected greatly by the treatment given, but that some cases nevertheless frequently persist in giving a positive reaction. In hereditary lues a positive reaction often persists in spite of most rigorous interference, or it may return to positive shortly after the cessation of treatment, so that it may be necessary to make frequent tests to determine whether further therapy is indicated. Vernes of the Institut Prophylactique of Paris claims that syphilis may be regarded as cured if the reaction remains negative for a period of eight months. While it is generally agreed among the profession that a positive reaction in a syphilitic case is an indication for additional treatment, it is not definitely established that the disappearance of the reaction is justification for the cessation of treatment, especially as the reaction may be quickly affected by therapeutic measures.

ARSENICAL TREATMENT

The influence of the treatment of syphilis with Ehrlich-Hata dioxydiamidoarsenobenzol or salvarsan, and various other similar arsenical compounds (neo-salvarsan, arsaminol, galyl, hectine, etc.) upon the serum reaction has been studied by various investigators.

Nichols treated rabbits and monkeys experimentally inoculated with syphilis and yaws by means of an intravenous injection of the arsenobenzol and

found that the spirochætæ disappeared within twenty-four hours and the serum reaction became negative within about four weeks after the injection.

Observations upon human subjects have been made by a large number of investigators on a vast deal of syphilitic material in various clinics and hospitals. The early literature on salvarsan is reviewed below, with special reference to the number of relapses and the effect of the treatment upon the Wassermann reaction. It may be mentioned that the statistics gathered by these early investigators differ in no wise from those which have been obtained by later workers.

Wechselmann treated 1250 cases and observed only 40 relapses. The serological study of Wechselmann's cases was conducted by Lange, who analyzed 268 cases in detail. They found that 153 out of 268 cases became negative in five weeks after the injection. There were 18 cases which were negative before and after the injection. The reaction was still positive in 97 cases at the end of five weeks, although more or less reduction in the strength of the reaction was noticed in 34 of them.

Michaelis treated 110 cases and observed 3 relapses. According to him the serum reaction may become negative within a period of from two to ten weeks after the injection. In a few instances the reactions became stronger than before the injection

while the clinical symptoms were fast disappearing under the influence of salvarsan. This last phenomenon has also been observed by Munk and Fraenkel and by Grouven.

Herxheimer treated 789 cases, among which 33 relapses occurred within a period of five months. Many of his cases were lost sight of before a negative reaction was obtained. In a limited number of cases which remained under his observation for some time the reaction became negative in 75 per cent of cases within fifty days after the injection. Bering reports that the reaction became negative in 40 cases after the lapse of five weeks or longer, while it remained positive in 26 cases.

Treupel, Halberstädter, Ledermann, Schlesinger, Bruhns, and Hoffmann noted very slow reduction or persistence of the reaction in most of their patients. On the other hand, Kromayer, Gennerich, Linser, Cramer, and others saw the reaction disappear in at least 50 per cent of cases within four or five weeks.

In the work just summarized the arsenobenzol was used either in a neutral or a slightly alkaline suspension and was administered intramuscularly by one group and subcutaneously by another group of investigators. In the beginning the dose was only 0.3 gm. (instead of 0.6 gm. as recommended later).

The intravenous administration of salvarsan was advocated by Schreiber, who compared the efficacy

of the arsenobenzol by giving it intramuscularly in
one series and intravenously in another series of
cases. Schreiber administered salvarsan intramuscu-
larly in 152 cases and observed 18 relapses, while in
565 cases treated by the intravenous injection there
was only one. In his earlier report Schreiber mentions
that the reaction disappeared in 50 per cent of cases
after the injection. He afterwards succeeded in
causing the reaction to disappear in every case by
continuing the intravenous injections as long as the
reaction remained positive. Géronne treated 220
cases by the intravenous administration of salvarsan
and observed 14 relapses. Of 77 cases of syphilis in
all stages the reaction remained negative in 37 for
at least eight weeks after injection, while in the
other 40 it became positive after having been nega-
tive for a brief period. In 9 out of 13 relapses the
reaction immediately became negative upon a second
injection.

The total number of cases available for a complete
serological analysis by the writer was 102. The quan-
titative determination of the Wassermann reaction
was carried out with the antihuman system in the
manner already described in connection with the
titration of syphilitic antibody (p. 101). The blood
was examined before the injection and then 1 day,
3 days, 1 week, 2, 3, 4, 6, 8 weeks, etc., after the in-
jection. Table XXXVI shows the serological conditions

TABLE XXXVI

	Number of cases	Before the injection of salvarsan — Number of syphilitic antibody units in 0.02 c.c. active serum										Average units	After the injection of salvarsan — Number of syphilitic antibody units in 0.02 c.c. active serum											Average units	Compared with the original (before injection)	Percentage of negative reactions after the injection
		1	2	3	4	5	6	7	8	9	10		<1	1	2	3	4	5	6	7	8	9	10			
Primary syphilis	12	2	4	3	2	1	..	..	..	..	..	2.66	5	3	1	..	..	..	..	..	..	..	..	0.54	1/5	40
Secondary syphilis	38	2	11	12	5	3	2	1	1	..	1	3.50	14	8	4	2	3	..	..	..	..	..	..	0.98	1/3.5	37
Tertiary syphilis	31	6	6	8	3	5	3	..	..	..	..	3.18	11	8	6	1	..	..	..	..	..	..	..	0.88	1/3.6	35
Latent syphilis	6	3	2	..	..	1	..	..	..	..	..	2.00	2	..	1	..	..	..	..	..	..	..	..	0.40	1/4	33
Hereditary syphilis	7	1	1	3	1	..	..	1	..	..	..	3.28	1	2	3	..	..	..	..	..	..	..	..	1.20	1/2.7	14
Cerebrospinal syphilis	6	2	2	2	..	..	..	..	..	..	..	2.00	1	2	3	..	..	..	..	..	..	..	..	1.35	1/1.5	0
Tabes	2	..	2	..	..	..	..	..	..	..	..	2.00	..	1	..	..	..	..	..	..	..	..	..	0.50	1/4	50
Total	102	16	28	28	11	10	5	2	1	0	1	2.66	34[1] 20[2]	24	18	3	3	0	0	0	0	0	0	0.83	1/3.2	33.7

These 102 cases are commonly designated as strongly positive reactions, but note that a strongly positive reaction can be caused by a number of the antibody units varying from 1 to 10.

The 48 cases indicated by brackets (47.5 per cent) without analysis would fall in the group of strongly positive reactions.

[1] Percentage of negative reactions, 33.7.
[2] Percentage of weakly positive reactions, 19.8.

of 102 cases before and after the injection of the arsenobenzol and furnishes us with many interesting facts with regard to the Wassermann reaction.

With regard to the varieties of the reactions in these 102 strongly positive specimens the reader will notice that in primary, secondary, tertiary and hereditary syphilis the average specimens contained more than one antibody unit, more frequently 2, 3, and 4 units. Among the specimens derived from secondary syphilis one with 10 units was encountered. On the other hand, the majority of specimens from latent syphilis contained 1 or 2 units. In cerebrospinal syphilis and tabes the antibody content was also comparatively low. Taking the average for different stages of syphilis in groups one finds that of the secondary to be the highest, followed by those of the hereditary, tertiary, primary, and latent syphilis. It may be recalled here that any specimen containing more than one antibody unit is capable of giving a complete inhibition of hemolysis, commonly known as strong positive reaction.

In the serological conditions which were created in the same series of cases after the injection of "salvarsan" we find a striking contrast to the conditions which existed before injection. After the treatment none of the sera contained more than 4 units, usually 1 or 2 and seldom 3 or 4. In 30 cases the reaction became negative, in 24 cases less than

one antibody unit remained, while the remaining 48 (47.5 per cent) sera still gave strong positive reactions. Superficially, these 48 cases might be taken as an evidence that the arsenobenzol had no influence upon them, but by quantitative examination the antibody content in these sera is found to be decidedly reduced, as will be seen in the table. Comparison of the average antibody units for each group of cases and also the ratios between the averages for the original and those after the treatment shows that the average for primary cases was reduced to 1/5, for secondary to 1/3.5, for tertiary to 1/3.6, for latent to 1/4, for hereditary to 1/2.7, for cerebrospinal to 1/1.5 and for tabes to 1/4 of the original antibody contents. This shows clearly that the arsenobenzol had the effect of reducing the antibody content in general.

With regard to the frequency with which positive reactions disappeared after treatment, we find that in 40 per cent of the primary cases, 37 per cent of secondary, 35 per cent of tertiary, 33 per cent of latent, 14 per cent of hereditary and 50 per cent of incipient tabes the reaction became negative, while in all cases of cerebrospinal syphilis it became somewhat weaker but still remained positive. The average of the negative reactions corresponds to 33.7 per cent of the total 102 cases.

The percentage of weak positive cases was 19.8 of

the total, but the reaction in these cases was progressively losing strength and may have fallen in due course of time into the category of the negative group or returned into the positive group if treatment was not effective.

The Relation Between the Clinical and Serological Findings. In order to determine what relation exists between the clinical data and the serological findings a brief summary of the clinical observations is presented in Table xxxvii.

TABLE XXXVII

LATENT CASES NOT INCLUDED

	Symptoms disappeared	Slow improvement	No improvement	Relapses
Primary syphilis.................	12[1]			
Secondary syphilis..............	30[2]	7	2	10
Tertiary syphilis.	19[3]	10	3	1
Hereditary syphilis..............	5[4]	2		
Cerebrospinal syphilis...........			6	
Tabes.........................		1[6]	1	
	66[5]	20[7]	12	11

[1] 5 became negative. [2] 14 became negative. [3] 11 became negative. [4] 1 became negative. [5] 31 became negative. [6] Became negative. [7] 1 became negative.

In 66 cases which responded favorably to the arsenobenzol, the clinical symptoms disappearing within a few weeks, the serum reaction became negative or less marked. The effect of salvarsan upon the clinical symptoms is far more prompt than upon the serum reaction. In a large number of cases the gen-

eral condition improves within a week, and not infrequently, within twenty-four hours after the injection. Healing usually progresses quickly, disappearance of lesions occurring within a few weeks. On the other hand, the degree of reaction of the serum decreases gradually, a considerably longer time being required for it to become negative. In this group 31 cases only were negative after treatment; in the remaining 35 the reaction was still positive to some degree.

In Table xxxviii are presented the data with regard to the time of disappearance of the reaction in cases of syphilis after the injection of salvarsan.

TABLE XXXVIII

SHOWING THE LENGTH OF TIME REQUIRED FOR POSITIVE REACTIONS TO BECOME NEGATIVE AFTER SALVARSAN TREATEMNT

	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks	Total
Primary syphilis.......		1	1	2		1			5
Secondary syphilis.....			3	5	3	2	1		14
Tertiary syphilis.......		2	3	4	2				11
Latent syphilis.........			2						2
Hereditary syphilis.....						1			1
Cerebrospinal syphilis..									0
Tabes................			1						1
		3	10	11	5	4	1		34

In 20 cases in which clinical improvement was very slow, the degree of serum reaction also diminished very slowly, and in some cases it became sta-

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tionary. In 2 cases of tertiary syphilis the clinical symptoms showed but little improvement, while the reaction with the blood became slowly but progressively weaker within two months. Six cases of cerebrospinal syphilis (Henderson) showed no improvement clinically, but the reactions were somewhat weaker after five weeks. In 2 cases of incipient tabes (Pedersen) the clinical and serological conditions improved in one and not in the other.

Ten relapses were observed in the 102 cases. Most of these were malignant or tertiary syphilis. The recurrence of the disease could always be detected by the return to positive of the reaction, which had previously been diminishing. In six cases of relapse salvarsan was reinjected with good results: one of the six had a second relapse. There were 3 in which salvarsan had no effect either upon the reaction or upon the symptoms. In 4 instances the reactions became somewhat stronger for a few days following the injection, then gradually disappeared.

Craig conducted a comprehensive study on the immediate effects of salvarsan treatment on the complement-fixation reaction (antihuman system). The results obtained by him are especially valuable because the patients (soldiers) were kept under constant observation, and tests could be made at any time. Under such favorable circumstances Craig examined 700 soldiers, and reported in September,

1911, an analysis of the results on 225 cases which had been observed for at least eight weeks after the administration of salvarsan, most of them for a longer period. 72.8 per cent became negative, and 27.1 per cent remained positive. Of the negative group 14.6 per cent suffered relapse; 43 remained negative for two months; 41 for two and a half months; 20 for three months; 7 for four and a half months; 11 for five and a half months; 7 for six months, and 6 for seven months. The reaction disappeared more readily in primary (80.6 per cent) than in secondary (74 per cent), tertiary (54.9 per cent), or latent syphilis (72.9 per cent). The number of relapses was also smaller in primary (12 per cent) than in secondary (15 per cent), tertiary (16.6 per cent) or latent cases (14 per cent).

The rapidity of disappearance of the reaction varied according to the stage of the disease. The tertiary cases became negative more rapidly than others, 9 of the 12 becoming negative within two weeks. In the secondary cases the reaction disappeared most frequently during the second, third and fourth weeks, the longest period being eight weeks. In the primary cases, about half became negative within two weeks, and the rest within five weeks. In latent cases the majority became negative during the second, third, fourth and fifth weeks, and no case became negative after six weeks.

The relation of the intensity of the reaction to its disappearance was also analyzed. Of the 119 cases with more than one antibody unit (complete inhibition of hemolysis) the reaction became negative in 66.3 per cent of which number 16.4 per cent relapsed. Of 71 cases with less than one and more than one-half antibody unit (inhibition at least 50 per cent) 80.2 per cent became negative, of which 12.2 per cent relapsed. Of 35 cases with less than one-half antibody unit (inhibition less than 50 per cent) 80 per cent became negative and 14.2 per cent relapsed. Nearly half of the cases giving a weak reaction (less than 50 per cent inhibition) were patients in the late secondary or tertiary stages of the disease in which the infection was still active, although they had been treated vigorously with mercury. One-fifth of the primary cases also gave a weak reaction and 71 per cent of this group became negative.

Craig gave special attention to the relation of the method of administration of salvarsan to the disappearance of the complement-fixation reaction. The methods used in the cases reported were as follows: Injection of the neutral suspension; intramuscular injection of the alkaline solution; intravenous injection, and combined intramuscular and intravenous injections.

While the disappearance of the reaction was more

rapid in the cases treated with the intravenous than with the intramuscular injection, yet the percentage of the relapses was strikingly higher in the former.

TABLE XXXIX

		Became negative	Re-mained positive	Relapse
		Per cent	Per cent	Per cent
1. Neutral suspension................	9	77.7	22.3	71.4
2. Alk. sol. intramuscular...........	150	78.6	21.4	6.7
3. Intravenous.....................	30	53.3	46.7	37.5
4. Combined intramusc. and intra-venous.......................	36	63.8	36.2	21.7

The effect of a previous mercurial treatment was also considered. It was found that the reaction disappears more rapidly in the mercurialized than in untreated cases, and the chances of relapse are less, although relapses have occurred in patients previously well treated with mercury. In the 225 cases reported, 110 patients had received no treatment previous to the use of salvarsan. Of these 74.5 per cent became negative; 25.4 per cent remained positive, and 12.2 per cent relapsed. Of 75 cases previously treated with mercury, 84 per cent became negative; 16 per cent remained positive, and 9.3 per cent relapsed. In 24 cases the intervals between the time when the reaction became negative and that when relapse occurred varied from 4 to 23 weeks, as follows:

4 weeks.......................................	2
5 weeks.......................................	4
6 weeks.......................................	3
7 weeks.......................................	4
8 weeks.......................................	1
9 weeks.......................................	1
10 weeks......................................	1
11 weeks......................................	2
12 weeks......................................	2
14 weeks......................................	1
16 weeks......................................	1
22 weeks......................................	1
23 weeks......................................	1
	—
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A great many of these relapsing cases had a strong fixation reaction before the treatment with salvarsan.

The careful analysis of Craig brought out clearly the close relation between the serum reaction and the therapy and diagnosis of syphilis. Craig and Nichols are inclined to consider that the absence of clinical and serological manifestations persisting for a period of over one year after the treatment with salvarsan indicates a cure of the disease. This view is shared by Vernes[1] (Paris) who regards a patient who remains well after treatment for a period of eight months without recurrence of symptoms or Wassermann reaction as permanently cured of syphilis. The cutaneous reaction with luetin may be used as another means of detecting the latent

[1] The perethynol reaction of Vernes consists in determining the capacity of a syphilitic serum to render the hemolytic component of fresh pig's serum inactive against sheep erythrocytes in the presence of a colloidal suspension of horse heart muscle lipoids.

focus of the infection in this class of patients, since it reveals latent syphilis in many cases in which the serum reaction or clinical symptoms are absent. A distinct cutaneous reaction was obtained with luetin in certain cases treated with salvarsan and mercury in which no clinical or serological signs of syphilis had been manifest for one year or longer.

CHAPTER XIII

Specific Complement-fixation in Syphilis

THERE are two facts, namely, the existence of so-called "antigen" in nonsyphilitic tissues and the occurrence of the Wassermann reaction in nonsyphilitic diseases (yaws, leprosy, malaria, etc.), which disprove indirectly that the Wassermann reaction is caused by true syphilitic antibodies and antigen (*Treponema pallidum*). The obstacle to separating conclusively the Wassermann reaction from the specific antibody-antigen fixation was the lack of pure cultures of *Treponema pallidum*. The pure culture furnishes us with a material in which we can obtain the *pallidum* substance free from any tissue lipoids which might produce the Wassermann reaction.

After cultivation of *Treponema pallidum* in 1910–1911, the writer immunized for several months a number of animals (rabbits, sheep) with the pure *pallidum* extract and tested their sera for the complement-fixation simultaneously with both the *pallidum* extract and the pure lipoids. These immune sera were found to bind complement with the *pallidum* extract, but not with the lipoids. On the other hand, the sera from rabbits with active experimental syphilitic orchitis bound complement with the lipoids, but not with the *pallidum* extract. The findings

showed that the immune sera contain the specific antibodies for the *pallidum* but not for the lipoids, and that in the sera of syphilitic rabbits (active stage) there is an abundance of the lipotropic substance capable of rendering complement inactive (so-called fixation) in the presence of certain lipoids, but too little specific antibody to bind complement with the *pallidum* antigen. This clearly demonstrated the difference between the Wassermann and the true antibody antigen reaction in syphilis.

The results obtained in experimental syphilis in animals hold good also for human syphilis, for the writer found that the sera derived from primary and secondary cases of syphilis almost never give a positive reaction with the *pallidum* antigen, and, if there is any, it is usually weakly positive. The same sera, as is well known, usually give a strong fixation with the lipoidal "antigen" (Wassermann reaction). While there is a striking absence of the specific antibodies (negative phase) during the active stage (primary and secondary) of syphilis, we find among the sera of patients suffering from the chronic course of syphilis (tertiary, latent, tardive congenital) some which contain varying quantities of the specific antibodies to be detected with the *pallidum* antigen. In this latter class of patients the Wassermann reaction may be positive or negative. Wassermann-negative sera from patients who have

undergone energetic treatment more frequently give a positive fixation with the *pallidum* antigen, owing possibly to a more vigorous formation of the specific antibodies. In general, however, the amount of the specific antibodies in human syphilis is remarkably small, and a complete inhibition of hemolysis is obtained infrequently, even when the serum is used in maximum quantity. On the other hand, the immune sera of rabbit or sheep give complete fixation with the *pallidum* extract.

The significance of a positive reaction with the *pallidum* antigen awaits final solution, yet it is not improbable that a positive reaction (the presence of the specific antibodies) is a favorable sign for the patient. It is desirable, indeed, to establish a definite relationship between the specific reaction and prognosis. In view of possible interest for serologists and clinicians the writer presents below a brief technique for making the fixation test with the *pallidum* extract.

The Pallidum Antigen. Pure cultures of several different strains of *Treponema pallidum* of various ages (*e.g.*, 7, 14, 21, 28, 35, 42 days) grown in the writer's special medium, are ground in a mill until the *pallida* are nearly disintegrated. The thick emulsion is then diluted with 0.9 per cent salt solution. It is first titrated for its anticomplementary property in the usual manner. The amount of complement (guinea-pig's fresh serum) to be used in the titration

is 0.04 c.c. (or 0.1 c.c. of 40 per cent dilution). If the *pallidum* extract inhibits the action of the complement in the dose of 0.2 c.c., for example, 0.1 c.c. or half of the inhibiting titre for the fixation test is employed. The antigen may be preserved on ice with the addition of 0.4 per cent phenol. The writer does not use an emulsion which has been standing over one week. It is wise to test its reliability by means of an immune serum (rabbit or sheep).

The Patient's Serum (Antibody). The serum of the patient is collected in the usual way (venepuncture, etc.). It should be previously heated to 56° c. for thirty minutes in a water-bath. This precaution is absolutely necessary because of the protein nature of the *pallidum* antigen (pp. 57–58); in the lipotropic or Wassermann reaction pure lipoids are employed, hence this distinction. For the test 0.1–0.2 c.c. of the inactivated serum is used.

The Complement, Amboceptor, and Corpuscle Suspension. As given in procedures described on pp. 60–67, 88.

The Technique for the Fixation Test. As indicated in the table on p. 95.

COMPLEX COMPLEMENT-FIXATION PHENOMENON

The clear distinction between the Wassermann and the specific fixation reaction in syphilis is brought out only through the use of pure lipoids on the one hand and a pure *pallidum* extract on the other. This

is no doubt a valuable distinction, inasmuch as the former indicates the activity of the infecting organism (*Treponema pallidum*) and the latter the immunity reaction on the part of the host. But when the complement-fixation test is made by means of an antigen containing both the active lipoids and the pallidum antigen, the result is capable of two different interpretations. It may have been a sign of the activity of the *pallidum* or that of the host against the pallidum, or a mixture of the two phenomena. The original aqueous antigen of Wassermann, Neisser and Bruck is likely to bring about this mixed phenomenon, because it contains both the lipoids and the *pallidum*. As a matter of fact, the rôle of the *pallidum* in the Wassermann reaction is very insignificant in the majority of instances, for the reason that whenever the reaction is very strong (as in primary or secondary syphilis) it is usually caused by the lipotropic substances alone and not by the specific antibodies. It may become of some importance only in the chronic cases. Yet, if one obtained a positive reaction with an aqueous antigen, he would be at a loss to determine whether the reaction was due to the lipotropic substance (Wassermann) or the specific antibodies or to both. This confusion does not occur in primary or secondary syphilis (p. 185), but it may be avoided by using pure lipoids and pallidum antigen separately. The specific antigenic

substance of the *pallidum* is insoluble in alcohol, hence an alcoholic extract does not fix complement either with an immune serum or with human serum containing *the specific antibodies*. From this fact it follows that an alcoholic extract of a fetal liver with *pallidum* contains no specific antigen and does not differ from an extract derived from a normal liver. Therefore, the Wassermann reaction, as is understood today, is nothing but a lipotropic fixation, as practically every serologist is now employing an alcoholic extract or a purified mass of lipoids.

While no practical application of the phenomenon of specific complement fixation in syphilis has been made, there is a possibility of further advance in this direction.

CHAPTER XIV

The Luetin Reaction

A CTIVE or passive immunity, the state of allergy, and numerous other phenomena, which have been so commonly observed and profitably studied in certain infectious diseases since the successful pure cultivation of their respective causative agents, are rather imperfectly understood in syphilis. Soon after obtaining pure cultures of *Treponema pallidum*, the writer began to study these questions experimentally. A phase of the study which is of interest not only to those who are engaged in experimental work but also to clinicians at large is the cutaneous reaction in experimental and human syphilis.

Stimulated by von Pirquet's discovery of a specific cutaneous reaction for tuberculosis, several investigators (Meirowsky, Wolff-Eisner, Tedeschi, Nobl, Ciuffo, Nicolas, Favre and Gauthier, Neisser and Bruck, Jadassohn, and Fontana) attempted to obtain a specific reaction for syphilis by applying extracts of tissues prepared from syphilitic fetal liver or chancre to the skin of syphilitic patients. Their results were, on the whole, and in spite of some encouraging indications, contradictory. Neisser and Bruck, however, found that a reaction similar to that produced with syphilitic extract can be obtained also with a concentrated extract of normal

liver. This peculiarity of the skin of syphilitics is ascribed by Neisser to what he calls the state of "Umstimmung" in the later stages of syphilis. He, nevertheless, expressed the hope that the reaction might possibly be improved by employing an extract of the *pallidum* free from tissue constituents, but it was not available at that time. Pure cultures of *Treponema pallidum* of different ages furnish the material for the purpose; in them we possess also the metabolism products of the organisms, which doubtless are important factors in establishing an allergic state in syphilitic subjects.

The Preparation of the Luetin. Pure cultures of several strains of the *pallidum* are allowed to grow for periods of six, twelve, twenty-four, and fifty days at 37° C. under anaërobic conditions. One set is cultivated in ascitic fluid containing a piece of sterile placenta, and the other in ascitic fluid agar also containing placenta. The lower portion of each solid culture in which a dense growth has occurred is cut out and the tissue removed. The agar columns, which contain innumerable spirochætæ, are then carefully ground in a sterile mortar. The resulting thick paste is gradually diluted by adding, little by little, fluid culture containing an enormous mass of the pure organisms. The dilution is continued until the emulsion becomes perfectly liquid. The preparation is next heated to 60° C. for thirty minutes in a

water-bath, and then 0.3 per cent tricresol is added. When examined under the darkfield microscope, numerous dead pallida per field may be seen. Cultures made from this suspension remain sterile, and with them no infection can be produced in the testicles of rabbits. The suspension is kept in a refrigerator when not in use. The name *luetin* has been adopted for this preparation.

In order to ascertain that the reaction produced with this suspension is not due solely to the introduction of the tricresolized culture medium alone, it is desirable to prepare a similar emulsion with uninoculated media to be used for control purposes.

Method of Application. In the case of rabbits, the hair on one side of the back is shaved off, an intradermal injection of luetin and one of the control suspension are made with a very fine needle in different spots, the amount injected being in each case 0.07 c.c. In the case of human subjects, the skin of the upper arm is selected as the site of injection. After sterilization of the skin with sublimate alcoholic solution, 0.07 c.c. of the luetin is intradermally injected into one arm and a similar quantity of the control suspension into the other.

EXPERIMENTAL BASIS FOR THE LUETIN REACTION

Before the skin test was applied to human subjects, several series of experiments were per-

formed on rabbits. The tests may be divided into five groups according to the treatment previous to the cutaneous tests. The first group comprised four rabbits which had received within five months twelve intravenous injections of *pallidum*-containing emulsions of material from syphilitic orchitis of rabbits, the last injection having been given two months previous to the application of the skin reaction. Ten different strains of *pallidum* were utilized in this series. The second group was composed of four rabbits which had been injected with similar emulsions, the difference being that the *pallidum* had been killed by heating the emulsions to 60° c. for thirty minutes before injection. The third group included twelve rabbits which were showing, four to six weeks after inoculation, syphilitic orchitis experimentally produced with several different *pallidum* strains. The fourth group consisted of four rabbits in which experimental syphilitic orchitis had been cured about four months previously by the intravenous administration of salvarsan. The fifth group consisted of eight normal male rabbits. The intradermic injections of the luetin and control emulsions were made on the same day in all the animals.

In the control (fifth) group, a very slight erythematous condition appeared twenty-four hours after inoculation at the sites of injection and disappeared within forty-eight hours, the skin resuming its nor-

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mal appearance. In the first and second groups of treated rabbits, the sites of injection of the control emulsion became normal within forty-eight hours, while the sites of injection of the luetin became distinctly red and indurated after forty-eight hours. The point of redness gradually spread and became enlarged to the size of a pea. This condition persisted from four to six days, when the reaction began to recede. The redness and induration disappeared in most animals within ten days. In one instance, a round sterile pustule developed on the fifth day at the site of each of two inoculations of the luetin (Plate 1). These pustules resembled variolous pustules in general appearance and healed with crust formation. The third group gave only slight reactions, which possibly were somewhat greater than those in the control rabbits, but were not, except in a few instances, distinct enough to be considered positive. The fourth group behaved like the control. No constitutional effects were observed even among the animals with positive cutaneous reactions.

THE TYPES OF CUTANEOUS REACTION IN MAN

The reaction was carried out on a large number of syphilitic and nonsyphilitic individuals by various investigators, and a summary of the results will be given later. Before considering the practical value of

PLATE I.

An actively immunized rabbit (No. 4), inoculated on June 14, 1911. The drawing was made on the sixth day after inoculation. Both pustules are produced by the luetin. The control site disappeared.

PLATE II.

Right.   Left.

Male, aged 23 years. Chancre four months ago, followed by rupio-papular erup-
tions, which have cleared up with some scars in certain localities. Treatment: three
intravenous injections of salvarsan up to the time of making the luetin test. Wasser-
mann reaction weakly positive. The left arm shows the luetin reaction on its fifth day;
the right is the control.

PLATE III.

Female, aged 16 years. Hereditary syphilis, and interstitial keratitis of six months' standing. Salvarsan injected five months ago without good results. Wassermann reaction positive. Inoculated on June 17, 1911. The drawing was made on the fifth day. Both sites of the luetin injection show a tendency to pustulation. Marked telangiectasis.

the luetin reaction, ~~however~~, we may note the types
of the reaction in man.

Normal or Negative Reactions. After applications
of the emulsions, both luetin and control, to about
fifty nonsyphilitic individuals, it was possible to de-
termine the variations and limitations of the reac-
tions which follow intradermic administration in the
skin of such individuals. In the majority of nonluetic
persons there appears, after twenty-four hours, a
very small erythematous area at and about the point
of injection. No pain or itching sensation is experi-
enced. The reaction gradually recedes within forty-
eight hours and leaves no induration. In certain
individuals, the reaction may reach a stage of small
papule formation (about 4 × 4 mm.) after twenty-
four to forty-eight hours, after which (within sev-
enty-two hours) it begins to subside. No induration
is left behind, although occasionally slight yellowish
pigmentation may result from mild ecchymosis.

Positive Reactions. According to the manner and
intensity with which the skin of syphilitic individuals
responds to the introduction of the luetin, one may
distinguish the following varieties of effects:

(A) *Papular Form* (~~Plates II and~~ III). A large,
raised, reddish, indurated papule, usually seven to ten
millimeters in diameter, makes its appearance within
twenty-four to forty-eight hours. The papule may
be surrounded by a diffused zone of redness and

show marked telangiectasis. The dimensions and the degree of induration slowly increase during the following four or five days, after which the inflammatory processes begin to recede. The color of the papule gradually becomes dark bluish red. The induration usually disappears within two weeks; in certain instances a trace of the reaction may persist for a longer period. This latter effect is usually met with among cases of secondary syphilis undergoing regular mercurial treatment in which there are no manifest lesions at the time of the skin test. Congenitally syphilitic children under the age of one year also show this reaction.

(B) *Pustular Form.* The beginning and course of this reaction resemble the papular form until about the fourth or fifth day, when the inflammatory processes still increase in intensity. The surface of the indurated, round papule becomes mildly edematous, and multiple miliary vesicles occasionally form (Plate iv). At the same time, a beginning central softening of the papule can be seen. Within the next twenty-four hours, the papule changes into a vesicle filled at first with a semi-opaque serum that later becomes definitely purulent (Plates iv and v). Soon after this, the pustule ruptures spontaneously or after slight friction or pressure. The margin of the broken pustule remains indurated, while the defect caused by the escape of the pustular content

PLATE IV.

Male, aged 29 years. Chancre seven months ago, followed by secondaries. Six intramuscular injections of salvarsan, the last about three months ago. Developed exudative chorioiditis six weeks ago. The right eye shows typical exudative chorioiditis; the left eye is free from it. Wassermann reaction negative. Inoculated on June 23, 1911. The drawing was made on the sixth day. Between the two large luetin pustules there is a faint trace of the injection of the control material.

PLATE V.

Left.   Right.

Female, aged 51 years. Became infected twenty years ago, and was treated moderately by Lassar in Berlin. Present symptoms: exostosis and stenosis of lacrimal canal. Wassermann reaction weakly positive. Luetin tt on July 11th. The left arm shows two well marked pustules at the sites of the luetin injection, while the right arm shows a faintly visible trace on the control site. The photograph was taken on the sixth day. (Dr. S. Pollitzer kindly took this photograph for me.)

becomes quickly covered by a crust that falls off within a few days. A small induration sometimes remains for a few weeks or even months, leaving a small keloid after healing. There is a wide range of variation in the degree of intensity of the reaction described in different cases, some showing rather small and others a much larger pustule. This reaction was found almost constantly in cases of tertiary and late hereditary syphilis, as well as in cases of secondary syphilis which had been treated with salvarsan.

(C) *Torpid Form.* In rare instances, the injection sites fade away to almost invisible points within three or four days, so that they may be passed over as negative reactions, then suddenly become active again after ten days, or even longer, and progress to small pustule formation. The course of the pustule is similar to that described for the preceding form. This type of reaction has been observed in a few cases of primary, secondary and congenital syphilis and quite frequently among the cases suffering from syphilitic affections of the central nervous system.

Neither in syphilitics nor in parasyphilitics did a marked constitutional effect follow the intradermal inoculation of the luetin. In most positive cases, a slight rise in temperature took place, lasting for one day. In several tertiary and tardive hereditary cases,

however, general malaise, loss of appetite, and diarrhœa were noted.

THE PECULIARITY OF THE SKIN OF SYPHILITICS

The susceptibility to traumatic irritations of the skin of certain individuals suffering from tertiary syphilis is well known and has been much discussed by dermatologists. To account for the phenomenon, Finger advanced the theory of superinfection, which presupposes that a trauma creates a spot of weakened resistance in the skin and that the syphilitic virus wanders thither from a hidden focus in the interior to set up the lesion. On the other hand, Neisser maintains that it is not the spirochæta that produces a syphilitic lesion after a slight trauma, because he failed to prove the presence of any spirochæta or infectious agent in the lesions; but he believes that it is due to a pathological condition of the skin itself, which he calls "Umstimmung." Among 315 cases of syphilis the writer observed 15 cases (1 secondary, 12 tertiary, and 2 hereditary) in which the sites of the control emulsion reacted quite intensely, but such reactions were usually less intense than those produced by the luetin in the same persons. Furthermore, in these cases the induration remained much longer at the sites of luetin injection than at the control sites. In view of

these results and of the additional fact that the majority of cases giving the positive skin reactions did not react to the control emulsion, the writer believes that the reactions set up by the intradermic inoculation of luetin are not due to the abnormal irritative condition of the skin alone, but to a specific allergic condition. It is, of course, possible that both conditions may be coëxistent in the same patient, although the occurrence of "Umstimmung" appears, from my experience, to be far less frequent than that of allergy. In carrying out the cutaneous reaction for syphilis (as for any other infectious disease), it is highly important to provide adequate control observations to determine whether the state of "Umstimmung" exists only in syphilis, or whether it may also occur in other diseases.

It has been reported by a few later investigators that certain nonsyphilitic persons react positively to the intradermal injection of the luetin when they have been given potassium iodide or some mercurial compounds *per os* during several days just previous to the application of the luetin. The reactions in this class of persons seem to be much more rapid, requiring only twenty-four to forty-eight hours to reach their maximum. This is not the case with the typical luetin reaction.

THE RESULTS OF THE CUTANEOUS REACTION IN MAN

The first series of tests were carried out on 642 individuals, comprising 315 syphilitics, 77 parasyphilitics and 250 controls. The results obtained in syphilitic individuals are summarized in Table XL. In cases of primary and secondary syphilis which had had insufficient treatment or none at all the skin reaction occurred only in a few instances. The reaction in the positive cases was always of the indurated papular variety. In one primary case, in which the skin test was applied before regular mercurial treatment was begun, a torpid reaction followed the administration of mercury. On the other hand, most of the secondary cases which had undergone mercurial treatment before the administration of salvarsan, and which remained without symptoms for some months after the salvarsan injections, gave striking and unmistakable reactions.

The results in tertiary and late hereditary syphilis present a striking contrast with those observed in the earlier stages of the disease. The intensity of the reaction to luetin in these cases is probably increased by the state of "Umstimmung," but the constancy with which it appears suffices to render it a valuable clinical diagnostic aid. It is in this stage that syphilis manifests itself in its most diverse and obscure forms. A gumma or other tertiary lesion on the external parts of the body offers but little diagnostic

TABLE XL

LUETIN REACTION IN VARIOUS SYPHILITIC CONDITIONS AND IN CONTROLS

LUETIN REACTION	Primary syphilis		Secondary syphilis				Tertiary syphilis				Congenital syphilis				Cerebro-spinal syphilis		Latent syphilis		Controls	
	Symptoms present		Symptoms present		Symptoms absent		Symptoms present		Symptoms absent		Under 1 year		Late cases		Symptoms present				Non-syphilitic cases	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
No antisyphilitic treatment	13																24			250
Slight mercurial treatment	12	1	2	25			12				5	18								
Regular mercurial treatment					14	3	31		29	1	1	3	15	1	5	5				
Salvarsan and mercurial treatment			1		42	12			22	3			9							
Total	25	1	3	25	56	15	43	0	51	4	6	21	24	1	5	5	24	0	0	250
Complete Total	26		99				98				52				10		30		250	

315

250

difficulty; but it is not always easy to ascertain the luetic nature of lesions of the internal or specific organs. In tertiary cases, moreover, the Wassermann reaction is frequently absent, especially after antiluetic treatment, hence it is important to find that the skin reaction is more constant than the Wassermann reaction in tertiary syphilis. Two causes for a negative Wassermann reaction in the tertiary stage may be distinguished. The first, which may apply also to latent syphilis, consists in the restraining power of the body upon the propagation of the *pallidum*, together with the neutralization of its injurious products through the formation of antibodies. The second cause, which also operates in some primary and secondary cases, arises from the inability of the body to respond to the *pallidum* stimulus by the formation of the substances on which the Wassermann reaction depends.

The luetin reaction in syphilis of the central nervous system is less constant than the Wassermann reaction, but, with energetic treatment the luetin reaction becomes more frequent. The cases of latent syphilis reported here were in most instances parents of individuals with inherited syphilis. Many of the mothers had, however, suffered miscarriages previous to, or after the birth of, their syphilitic children.

The controls, including about 250 persons suffering from various diseases of nonsyphilitic nature,

tuberculosis, leprosy, pneumonia, typhoid fever, psoriasis, malaria, alcoholic psychosis, dementia præcox, gonorrhea, chancroid, brain tumor, eczema, epithelioma, carcinoma, etc., all gave a negative luetin reaction.

The results obtained in parasyphilitic cases were inconstant. Of 72 cases of general paralysis 45 reacted positively. In a few instances pustules were formed, but 27 gave no reaction. Of 5 cases of tabes, three reacted positively. Among 35 controls of this series, consisting of 15 cases of dementia præcox, 6 of alcoholic psychosis, and 14 of other forms of

TABLE XLI

	Number of cases		Symptoms present		Symptoms absent		Total	
			Luetin	*Wassermann*	*Luetin*	*Wassermann*	*Luetin*	*Wassermann*
Primary syphilis	4	Positive reaction	0	3			0	3
		Negative reaction	4	1			4	1
Secondary syphilis....	35	Positive reaction	20*	25‡			20	25
		Negative reaction	15†	10			15	10
Tertiary syphilis......	17	Positive reaction	13	8	4	2	17	10
		Negative reaction	0	5	0	2	0	7
Latent syphilis.......	5	Positive reaction	2	1	3	0	5	1
		Negative reaction	0	1	0	3	0	4
Congenital syphilis...	2	Positive reaction	1	1	1	1	2	2
		Negative reaction	0	0	0	0	0	0

* Under regular treatment. † No or slight treatment.
‡ The majority only slightly treated.

TABLE XLII

CONTROL CASES

	NUMBER OF CASES	LUETIN POSITIVE	REACTION NEGATIVE
Acne vulgaris..............................	9	1 *	8
Alopecia areata...........................	3	. .	3
Bromide eruption.........................	1	. .	1
Carcinoma................................	6	. .	6
Darier's disease...........................	1	. .	1
Eczema (various forms)...................	15	. .	15
Erythema multiforme.....................	4	. .	4
Erythema toxicum........................	3	. .	3
Herpes zoster.............................	5	.	5
Ichthyosis	2	. .	2
Keloid....................................	2	. .	2
Lupus erythematosus......................	6	. .	6
Molluscum contagiosum...................	1	. .	1
Pityriasis rosea...........................	2	. .	2
Psoriasis..................................	14	. .	14
Sarcoma (Kaposi).........................	1	. .	1
Scabies...................................	10	. .	10
Sycosis...................................	3	. .	3
Tinea versicolor..........................	2	. .	2
Trichophytosis............................	4	. .	4
Tuberculosis pulmonalis...................	12	. .	12
Xanthoma................................	2	. .	2
	108	1	107

* A woman of forty years of age with a history of two miscarriages was also a chronic alcoholic subject. The patient refused the Wassermann reaction which would have been of no value because of her constant usage of alcohol. Syphilis cannot be excluded from this case.

psychoses, 4 reacted positively, and these were cases of dementia præcox. These patients were adults, and 2 of them gave positive Wassermann reactions. The writer believes, therefore, that they had suffered either from congenital or acquired syphilis.

A large series of dermatological cases were studied

by Orleman-Robinson, whose results are quoted in Tables XLI and XLII.

Nobl and Fluss examined 100 dermatological cases at the "Wiener allgemeine Poliklinik," and made the following preliminary report:

TABLE XLIII

Luetin re-action	Pri-mary stage	Second-ary stage	Gum-matous stage	Late latency	Para-syphilis	Heredi-tary syphilis	Con-trols
Marked.....	2	21	1	9	2	1	3*
Weak.......	1	15		3		1	3*
None.......	4	26		4		1	3*
Total.....	7	62	1	16	2	3	9

* 4 out of the 9 control cases gave positive Wassermann, 1 case not tested.

These authors point out that they did not have large numbers of tertiary and latent cases in which the luetin reaction is expected to come out more constantly positive. They state also that from those control cases which responded to the luetin test a possibility of existing luetic infection is hard to rule out; and there were, indeed, 4 cases giving positive Wassermann reaction among the total 8 (1 case not tested for the serum reaction) examined. The luetin reaction was negative in three out of the four Wassermann negative cases.

Cohen applied the luetin test to ophthalmological cases, and obtained the following results:

TABLE XLIV

Name of disease	Number of cases	+ Clinical manifestations	−	Recently treated	Not recently treated	+ Wassermann	−	+ Luetin	− reaction
Interstitial keratitis.........	20	12	8	12	8	12	8	11	9
Kerato-iritis...............	3		3	1	2	2	1	2	1
Keratitis marg. profunda....	1		1		1		1	1	
Keratitis neuroparalytica....	1		1		1		1		1
Keratitis disciformis........	1		1		1	1			1
Acute iritis................	4	3	1	1	2	2	2	4	
Iridocyclitis (acute).........	3	1	2	2	1	1	2	2	1
Iridochoroiditis.............	1	1			1	1		1	
Chorioiditis exud. (acute)...	1		1		1		1	1	
Iritis serosa................	1		1		1		1		1
Scleritis...................	1		1		1		1		1
Optic neuritis..............	3	2	1	1	2		3	3	
Opt. atrophy (prim. 3, sec. 2)	5	1	4	1	4	5		4	1
Chorioretinitis.	1	1		1			1	1*	
Retinitis proliferans	2		2		2		2		2*
Detachment of retina	1		1		1		1		1*
Detachment of retina (traumatic)....................	1		1		1		1		1
Retinitis prolif. (traumatic)..	1		1		1		1		1
Macular chorioiditis........	1		1		1		1		1
Abducens paralysis.........	2	2		2		1	1	2	
Ophthalmoplegia externa....	1		1		1		1	1	
Thrombosis, cent. art. inf. br.	1		1		1		1		1
Anisocoria.................	1		1		1	1		1	
Stenosis lachr. canal........	1	1		1		1		1	
Puls. exophthalmos (idiop.)..	1		1		1	1		1	
Orbital tumor..............	1		1	1			1		1
	60	24	36	24	36	28	32	36	24

* Tuberculosis test +.

A { I. Cutan. test +, clin. evid. +, Wasserm. +, 12 cases
    { II. Cutan. test −, clin. evid. −, Wasserm. −, 16 cases

                                                    28 cases

B { I. Cutan. test +, clin. evid. −, Wasserm. −, 3 cases*
    { II. Cutan. test −, clin. evid. +, Wasserm. +, 11 cases†

                                                    14 cases

C { I. Cutan. test +, clin. evid. +, Wasserm. −, 2 cases
    { II. Cutan. test +, clin. evid. −, Wasserm. +, 6 cases
    { III. Cutan. test −, clin. evid. −, Wasserm. +, 7 cases‡
    { IV. Cutan. test −, clin. evid. +, Wasserm. −, 3 cases§

                                                    18 cases

                                                    60 cases

* These 3 cases may belong to latent syphilis in which no clinical or sero-logical manifestations are present.

† These 11 cases belong to untreated or only slightly treated early syphilis.

‡ These 7 cases may belong to an early latent stage in which the clinical symptoms had disappeared, but the luetin reaction had not yet developed.

§ These 3 cases belong to an anomalous type of patients in whom the Wassermann develops very slowly or may remain altogether absent. The luetin reaction had not yet developed.

The following cases are cited here from Cohen's observations as they illustrate the practical value of the luetin reaction in this class of patients.

1. *Orbital Tumor.* A married woman, thirty-one years of age, exhibited unilateral exophthalmos, orbital periostitis, ocular paralysis and keratitis lagophthalmos. Antiluetic treatment had resulted in no improvement. The Wassermann was negative. The luetin test was negative. Patho-logical examination of the specimen following the Kronlein operation for orbital tumor showed it to be a fibro-endothelioma.

2. *Case of Pulsating Exophthalmos, Bilateral.* A married man, forty-three years of age, whose wife had had three miscarriages, had exhibited eleven months previously a protrusion of the right eyeball; five weeks later the left eye had also begun to protrude. There was also paralysis of both external recti and anesthesia of the cornea of both eyes. Pupillary reac-tions normal. Visual fields normal. Vision markedly diminished, more on the right side than on the left. Veins of both upper lids congested, also the veins of both ocular conjunctivæ. Ophthalmoscopic examination showed dilatation and tortuosity of the retinal veins on the left side, but the right fundus was normal. A distinct bruit was heard over the left eyeball and left temple. Wassermann and luetin reactions both positive.

Four months before the patient was first seen he had begun to complain of roaring and buzzing noises in the head and of severe frontal headaches; but there were no other symptoms. No serious previous illness or trauma. The patient denied syphilis.

After antisyphilitic treatment for two months the exophthalmos in the right eye disappeared, the left eye improved somewhat; the paralysis of the external recti and the corneal anæsthesia disappeared on both sides; the venous engorgement in the upper lids disappeared on the right side and diminished on the left, but the congestion of both ocular conjunctivæ persisted. Vision improved from 20–200 to 20–40 in the right eye, and from 20–50 to 20–30 in the left eye. The symptoms, viz., the buzzing and roaring, disappeared entirely and the general condition improved markedly. The Wassermann and luetin tests were both still positive.

3. *Abducens Paralysis.* Male, forty years of age, had acquired syphilis three years previously, and had been energetically treated with injections of salicylate of mercury since. Diplopia for three months. Wassermann negative. Luetin reaction positive. Eye condition greatly improved after salvarsan and mercury treatment.

4. *Acute Iridocyclitis, Unilateral.* Male, twenty-nine years of age, had had a chancre seven months previously, followed by secondary manifestations. Treated with six intramuscular injections of salvarsan, each 0.3 gm. Notwithstanding the treatment eye symptoms developed. Wassermann repeatedly negative, luetin reaction strongly positive.

The foregoing series of cases, though limited in number and variety, confirms practically all essential points brought out by various investigators and shows that the luetin reaction is specific for syphilis and occurs most constantly and intensely during the period of tertiary syphilis. In primary or secondary stages the luetin reaction is usually absent or very mild, while it may become more intense after energetic treatment. In the congenitally syphilitic it is less marked in infancy and becomes more pronounced and constant later.

Later investigations of Wolfsohn on the luetin reaction made at the Johns Hopkins Hospital, in

the medical division under Professor L. F. Barker, are particularly interesting and valuable, as the diseases studied by him were chiefly internal, those in which syphilis may play an important part in etiology and in which a specific diagnostic test may be of practical value.

Wolfsohn's analysis and discussion of his cases are quoted below, as they bring out many interesting and instructive points regarding the luetin reaction in its relation to clinical manifestations and the Wassermann reaction.

"*Secondary Syphilis.* Two cases of secondary syphilis, both in the maculopapular stage, were inoculated. In each case the Wassermann reaction was positive and each patient had been given 0.4 gram salvarsan intravenously twenty-four to forty-eight hours before the test was given. Both tests were positive. In the first case the salvarsan had been administered only twenty-four hours before, but the reaction to the luetin, although delayed till the fifth day, was definitely positive. No constitutional symptoms or reaction (*Umstimmung*) over the site of injection of the control emulsion were noted.

"*Tertiary Syphilis.* This series includes six cases, in which the Wassermann reaction was positive in five. The sixth patient had had 0.6 gm. of salvarsan one year before admission and his test proved negative both for blood-serum and cerebrospinal fluid at

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this time. The Wassermann was positive in 1911, however. The luetin reaction in this case was positive. In the first case of this series the luetin reaction was negative, though the Wassermann was positive. This case was not followed longer than ten days.

"The reaction in four cases of luetic periostitis was of the violent variety and showed early pustule formation and rather marked reaction on the control side (*Umstimmung*). Two of these complained of tender and painful arms, and one developed enlargement of the axillary glands. All these manifestations disappeared in the following forty-eight hours.

"It is interesting to note that, in one case, two weeks after subsidence of the local manifestations there was a flare-up at the site of the injection of luetin. A pustule formed and rapidly disappeared, leaving no other signs than slight desquamation and pigmentation.

"The rapidity with which the reaction becomes manifest, and the ease with which it is interpreted, together with the almost constant development of it, in this stage of the disease, makes it a most valuable aid in diagnosis. It not only checks up the Wassermann reaction, but it may be indeed supplementary to it, especially in the treated cases, in which the Wassermann reaction not infrequently may be negative.

"*Parasyphilis.* If one accepts the newer conception of parasyphilis he will include not only tabes and general paresis, but those cases which show vascular changes of luetic origin, *e.g.*, cases of syphilitic mesaortitis and aortic aneurysms. Therefore, included in this series are 19 cases, seven of which are of central nervous system affection, and twelve of cardiovascular disturbance.

"A careful analysis of these cases shows the great value of the luetin test. Of the former cases all seven gave positive tests, while the Wassermann test was negative in six.[1] One of these, a tabetic, had a positive Wassermann reaction one year previously and had had in the interim three doses of salvarsan, but the cerebrospinal fluid still gave a positive Wassermann test. The luetin reaction in this case was violent, the reaction over the site of control injection (*Umstimmung*) being especially noticeable, as it was also in five of these cases.

"Another tabetic, who showed no other signs than diminished knee-jerks and sluggish pupils, developed remarkable constitutional symptoms, beginning 30 hours after inoculation and lasting four days. These symptoms consist of fever as high as 103° F., pain in the abdomen resembling crises, with nausea and

[1] Of course one must keep in mind the limited number of cases that the luetin test has been tried on; the result, however, even in this small series has been striking.

vomiting. The reaction was violent and *Umstim-mung* was present. The Wassermann reaction was negative, both for blood-serum and cerebrospinal fluid in this case. This patient had never received treatment previous to this time.

"Of the twelve parasyphilitics showing cardiovascular lesions, eleven gave positive luetin reactions and one reacted negatively. Only seven showed positive Wassermann reactions. The one case giving a negative luetin test had a positive Wassermann reaction. But six of this series giving negative Wassermann reactions gave positive luetin tests. It is in this group of cases in which great care must be exercised, as one might readily consider the test negative if it were not watched for some time. Six of these twelve cases showed reactions of the torpid form, no sign of the reaction appearing till the 9th day, and in one case of abdominal aneurysm, which gave a positive Wassermann test, there was no manifestation until the 28th day. The luetin reaction will prove of great value in this type of case, if properly followed, because the Wassermann tests are relatively inconstant. *Umstimmung* is infrequent here as compared with cases of parasyphilis of the central nervous system. No other cases showing constitutional symptoms were noted.

"*Latent Syphilis.* This stage of syphilis shows a remarkable constancy of results with this test. In

the twenty-three cases of latent syphilis tested all gave positive reactions. The Wassermann reaction in this series was positive only in ten cases. In three of these cases the Wassermann reaction with the cerebrospinal fluid was also negative.

"Some of the cases reported in this group denied having contracted syphilis, but after the luetin test was found positive, in several instances the patients confessed having had a primary lesion or secondary manifestations. The test was applied in 4 cases of pregnancy, three of which were completed by the birth of macerated fetuses and the fourth by the birth at term of a still-born infant. All four patients denied infection. The three former patients reacted 'violently' to the luetin and showed *Umstimmung*. The Wassermann reaction was positive and an ana- tomical diagnosis of syphilis in the infant was made in each case. The fourth case gave a negative luetin reaction, the Wassermann reaction was negative, and no signs of syphilis could be discovered in the dead infant. No constitutional disturbances were noted in these cases.

"Of the remaining nineteen cases in this series, nine showed *Umstimmung*. One patient suffering from carcinoma of the head of the pancreas, who had a chancre in 1865, showed a delayed luetin test, but gave a negative Wassermann reaction.

"*Constitutional Symptoms.* Constitutional symp-

toms consisting of tender axillary glands, tender arms, tachycardia, abdominal pains, bone pains, and nausea were present in four of the cases, but these symptoms were usually of only 24 to 48 hours' duration and caused no alarming discomfort to the patient. One case showed a second hemorrhagic pustule two weeks after the first manifestation had subsided.

"Thus latent syphilis, so difficult to diagnose clinically and in which the Wassermann reaction is not of assistance in over 40 to 70 per cent of the cases, showed a positive luetin reaction in all the cases tried. It seems that the luetin reaction will prove of even greater value in the diagnosis of this group than in the tertiary stage, in which the Wassermann reaction is more constantly present. The effects of treatment on the results of the luetin test in latent syphilis have not been significant.

"*Umstimmung.* In reviewing the whole series of one hundred and fifty cases, another interesting fact was noted, namely, that in many cases of tertiary and of latent syphilis the site of the control injection showed almost as marked a reaction as developed about the point where the luetin was injected. This, as was pointed out by Neisser and Bruck, and as seems to be confirmed by Noguchi and this series of tests, appears to be due to the susceptibility to trauma of the skin of syphilitics late in the disease (*Umstimmung*), for not one of the seventy control

patients reacted on the side in which the control emulsion was injected. . . .

"The cases exhibiting the most marked 'control' reactions (*Umstimmung*) were those suffering from syphilis in its later stages. The inconstancy of this phenomenon in all probability precludes its being of an allergic nature, which Noguchi, I believe, has so well shown the principle of the luetin reaction to be."

Wolfsohn drew the following conclusions:

"1. The luetin reaction is specific for syphilis.

"2. The reaction is found of greatest value in the latent and tertiary stages of the disease.

"3. In some treated cases of secondary syphilis the reaction is positive.

"4. In parasyphilitics with the cardiovascular manifestations of the disease the reaction may be delayed for from 9 to 30 days.

"5. The luetin reaction is helpful in the diagnosis of latent syphilis in pregnancy.

"6. The state of *Umstimmung* is well brought out in the tertiary and latent forms of syphilis."

In pediatrics the luetin reaction seems to find its useful application. For example, Brown, working under Professor L. Emmet Holt, at the Babies' Hospital, New York, obtained a positive reaction in 30 (90 per cent) out of the 33 children suffering from congenital syphilis, while the remaining 3 cases gave doubtful reactions. In 73 nonsyphilitic chil-

dren there was no positive reaction. In 15 positive cases the reaction was of pustular form, and the other 15 showed the inflammatory nodules. When arranged according to age the reactions were found to be as follows:

TABLE XLV

	Positive reaction	Doubtful reaction
1 to 3 months.	12	3
3 to 6 months.	8	
6 to 12 months.	6	
1 to 4 years.	4	
	—	—
	30	3

It was also found that the more energetic the treatment the more distinct was the luetin reaction. The 3 infants who gave the indecisive reactions had not been under antisyphilitic treatment, while the 30 positive cases had been having treatment either with mercury or salvarsan or both in combination for some time.

The varieties of control cases studied by Brown are shown in the following list. The Wassermann as well as the luetin reaction was uniformly negative in these cases.

The results of the study by Brown establish the specificity of the luetin reaction for syphilis in pediatric cases.

TABLE XLVI

NONSYPHILITIC CASES, BABIES' HOSPITAL

No. cases	Diagnosis	
1	Scleroderma	The Wassermann and luetin reactions both negative in all cases
2	Spastic diplegia	
4	Marasmus	
2	Cretinism	
13	Bronchopneumonia	
2	Mongolian idiocy	
2	Eczema	
1	Bronchitis	
1	Retropharyngeal abscess	
7	Lobar pneumonia	
16	Infant feeding	
3	Tetanus	
3	Gastro-enteritis	
1	Pemphigus neonatorum	
1	Scarlet fever	
3	Empyema	
1	Tuberculous meningitis	
1	Adenitis	
1	Still's disease	
2	Rachitis	
1	Nevus	
1	Cleft palate	
2	Hernia	
2	Congenital heart disease	

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EFFECT OF TREATMENT UPON THE LUETIN REACTION

From what has been presented in the foregoing paragraphs it is quite evident that the luetin reaction remains but little affected by the usual intermittent mercurial treatment in a large number of cases. As already stated, the allergic condition may be brought out by the usual mercurial or any other antisyphilitic

treatment in those cases where it has not yet appeared. According to the writer's observations, salvarsan, either alone or combined with mercury, appears to exert the greatest influence, not only in inducing the development of the allergy, but also in removing it after it has developed. The explanation for this remarkable feature of salvarsan lies, no doubt, in its powerful spirochetocidal property, by virtue of which it destroys large numbers of the pallidum, thus inducing the allergic condition in earlier stages of syphilis, and then, if successful, annihilates all pallida, thus removing the source of the condition.

Although there can be no dispute about an accomplished cure with the mercurial treatment in a certain percentage of cases under favorable conditions, yet the successes are rather few. In the case of salvarsan treatment, there are a certain number which are definitely cured. The absence of the clinical and serological signs of syphilis during a period of one year indicates a possible cure, but, since these signs are often absent in latent cases, one may well hesitate in pronouncing these cases as cured. The luetin reaction may become an aid in settling this important question. As already stated, the luetin reaction alone cannot decide the point, but combined with other means of diagnosis it is bound to throw more light on the problem.

Kämmerer, working at the medical clinic of Prof-

essor von Müller, at Münich, Germany, subjected to trial the luetin submitted to him by the writer in a number of cases suffering from various diseases, and obtained results which are in the main confirmatory of those obtained by other investigators. Kämmerer found the reaction to be specific for syphilis although he obtained a lower percentage of positive reactions than did others. This is a highly important factor, as the value of the luetin rest depends upon its specificity.

CHAPTER XV

Examination of Cerebrospinal Fluid

THE several methods in use for detecting patho-
logically increased proteins in the cerebrospinal
fluid may be divided into two general groups ac-
cording to whether or not heat is required for their
performance. The group in which heat is necessary
comprises two reliable methods, the butyric acid test
and a test recently proposed by Amoss. This group
will be discussed first, and later details will be given
of a non-heating method recently worked out by the
writer and Miss E. B. Tilden.

BUTYRIC ACID TEST

The basis for this reaction was the writer's finding
that in the majority of syphilitic sera and cerebro-
spinal fluids there is a definite increase in the euglob-
ulin fraction. While there was no strict parallelism
between the increased globulin fraction and the
strength of the Wassermann reaction, there was
agreement among most of the specimens tested. In
a few instances only the Wassermann reaction was
not associated with any increase of protein.

In attempting to find a method by which the
increase in protein content could be detected, the
writer found that by first separating the globulin

fraction of the serum by fractionation and redissolving it in isotonic sodium chloride solution it could be easily precipitated out by the addition of a dilute solution of certain acids. Hydrochloric, acetic, proprionic, lactic, and butyric acids all produced the precipitation, but the differentiation between syphilitic and nonsyphilitic specimens was best obtained with butyric acid. Heating was avoided in this procedure, because it caused dense precipitation of globulin irrespective of origin. Later this test was found to be of no practical value for the detection of syphilis, because it only indicated the increased globulin in the serum regardless of whether it was due to syphilis, Hodgkin's disease, tuberculosis, or other conditions. The Sachs-Georgi reaction recently introduced also depends upon the precipitation of cholesterinized lipoids by a certain fraction of globulins in the blood serum. The reaction is so adjusted that sera from nonsyphilitic cases give a slight or no precipitate, while those derived from syphilis give a distinct precipitation. All observers agree that a number of nonsyphilitic cases give a positive reaction by this test, as would be expected in a test which depends upon the increase of globulin. Moreover, certain syphilitic sera show no increase in globulin and give a negative Sachs-Georgi reaction.

The butyric test was next applied to cerebrospinal fluid, but no precipitation was obtained. It

was found, however, that if the mixture was first heated to boiling, the excess of acid gradually reduced by adding sodium hydroxide solution, and the mixture boiled again, a copious precipitate resulted in the case of specimens with increased protein content. The method is as follows: 0.2 c.c. of spinal fluid is measured into a small tube and to it is added 0.5 c.c. of a 10 per cent solution of butyric acid in 0.9 per cent sodium chloride. The mixture is heated to boiling, 0.1 c.c. of normal (4 per cent) sodium hydroxide solution is added, and the whole again heated to boiling. The tube is then allowed to stand for 30 to 60 minutes. It is necessary to take the precaution to employ for this test only cerebrospinal fluid entirely free from blood, which itself gives a precipitate.

The presence of an increased content of protein in the cerebrospinal fluid is indicated by the appearance of a granular or flocculent precipitate, which gradually settles to the bottom of the tube, leaving a clear, supernatant fluid. The velocity and intensity of the reaction vary according to the quantity of the protein contained in a given specimen. The greater the amount of protein, the more quickly and distinctly the reaction appears. The granular precipitate appears within a few minutes in a specimen containing a considerable increase in protein, while one hour may be required to obtain a distinct reaction

in specimens containing less protein. A specimen which does not show a precipitate at the end of two hours may be regarded as negative.

This reaction appears regularly in the cerebrospinal fluid of the patients with syphilitic and parasyphilitic affections, and also in all cases of inflammation of the meninges caused by such micro organisms as *Diplococcus intracellularis*, pneumococcus, influenza bacillus, tubercle bacillus, etc. These acute inflammatory infections are of course readily differentiated from the syphilitic affections.

According to the investigations of Flexner and his co-workers, Lewis and Clark, the cerebrospinal fluid of man and of monkeys suffering from acute poliomyelitis contains an abnormally large amount of proteins and gives a positive reaction to the butyric acid test. It was found that the increase of the proteins is one of the earliest symptoms of this disease and reaches the maximum just before the paralytic symptoms develop. The test was employed by Flexner in his early experiments to determine the result of inoculation of the poliomyelitic virus into monkeys. He also discovered a certain number of abortive cases of poliomyelitis in man by means of the butyric acid test and cytodiagnosis, eliminating (by careful clinical data and other differential diagnostic means) all other diseases giving the same reaction together with lymphocytosis. Specimens of cerebro-

spinal fluid from cases of lethargic encephalitis show a slight increase in protein content.

Normal cerebrospinal fluid gives with the butyric acid test a slight opalescence and sometimes a marked turbidity, but the granular precipitate does not occur at all or only after six hours or longer.

AMOSS'S TEST

Because of the unpleasant odor of butyric acid, Amoss devised a method in which he employed a solution containing 0.5 c.c. of glacial acetic acid and 3 gm. of primary sodium phosphate in 100 c.c. of distilled water. 0.6 c.c. of this reagent is mixed with 0.2 c.c. of cerebrospinal fluid and the mixture boiled for six minutes in a water-bath. The results are almost identical with those of the butyric acid test, although in some weak reactions the test is perhaps less sensitive.

TEST WITH LIPOIDAL REAGENT

Among the non-heating procedures, that recently worked out by the writer and Miss Tilden is the simplest and has been found to give entirely satisfactory results.

The components of the reagent are: (1) alcoholic extract of acetone-insoluble tissue lipoids (Solution 1), and (2) an aqueous solution containing in 1000 c.c. 0.5 c.c. acetic acid, 10 c.c. of a saturated solution

ERRATUM

Page 224, line 10, read "0.05 c.c." for "0.5 c.c."

of picric acid in absolute alcohol, 1.5 gm. acid potassium phosphate, and 4 gm. sodium chloride (Solution 11).

Preparation of Solution 1. Beef heart is ground in a sausage machine, then completely dried by a fan over a heater. 300 gm. of the dried substance are extracted with 1 liter of acetone for five days at room temperature, with daily shakings. The acetone is then discarded, the mass of solids freed from acetone by evaporation and then extracted with 1 liter of absolute alcohol for five days at room temperature. The golden yellow alcoholic extract, which contains acetone-insoluble lipoids (and which has been found to be an excellent antigen in the complement fixation test for syphilis,[1] p. 72), is separated from the dried muscle and tested for its suitability as the reagent. The criterion of usefulness is the transparency of a mixture of the alcoholic extract and Solution 11 in a ratio of 1 to 9. If a marked opalescence bordering on opacity is produced, the extract is unsuitable.

[1] It may be stated that the concentration of the acetone-insoluble lipoids in such an extract is approximately 1.2 per cent. The peculiarity of the dry extraction product is that, notwithstanding its high concentration of lipoids, the mixture of the extract and 0.9 per cent saline solution brings about only slight turbidity, even when the latter is added drop by drop with vigorous shaking to a 1 : 10 dilution. This comparatively clear solution is seldom anticomplementary or hemolytic, but usually possesses a high antigenic value. While the complement-fixing power of other antigenic preparations is almost proportional to the degree of dispersion of lipoidal molecules in suspension, that general rule apparently does not apply to this preparation.

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As a rule a satisfactory solution—faintly opalescent, but almost transparent—is obtained.

Preparation of Solution II. It is convenient to prepare a stock solution of the acids and salts in distilled water as follows: 1.5 gm. of acid potassium phosphate (KH_2PO_4) and 4 gm. of sodium chloride are dissolved in 990 c.c. of distilled water containing 0.5 c.c. of glacial acetic acid. Finally, 10 c.c. of a saturated solution (approximately 3 per cent) of picric acid in absolute alcohol is added. If the solution is not to be kept in the refrigerator it is desirable, in order to prevent any fungus growth, to make it up in ten times the strength in which it is to be used, that is, to dissolve the salts in 90 c.c. of distilled water containing 0.5 c.c. glacial acetic acid. At the time of use 1 part of this solution should be mixed with 7.9 parts of distilled water and 0.1 part of the picric acid solution; 1 part of the lipoidal solution is then added.

Preparation and Preservation of the Reagent. To 9 parts of Solution II is added gradually, mixing by gentle agitation, 1 part of the alcoholic lipoidal extract (Solution I), the resulting mixture being faintly opalescent, almost transparent. (If the process is reversed and Solution II poured into Solution I, a turbidity results which makes the reagent unsuitable.) The mixture in this form has been found to remain unchanged for a period of several weeks in the

refrigerator and will perhaps prove not to be subject to deterioration, but as the two solutions may be preserved separately for an indefinite period the reagent can be made up freshly from time to time as needed.

It must be mentioned that the ordinary alcoholic extract of beef heart with or without the addition of cholesterin (so-called syphilitic antigens used by some serologists) does not give a useful reagent, but one too sensitive, readily producing precipitation even when mixed with normal spinal fluid. An alcoholic extract of the dried beef heart muscle without preliminary acetone extraction is likewise unsuitable, owing to the presence of certain acetone-soluble lipoids (cholesterin, etc.). The addition of cholesterin to a suitable alcoholic extract of acetone-insoluble tissue lipoids renders the latter too sensitive and therefore worthless as the reagent.

A differentiation can be made between pathological and normal spinal fluids without the addition of the salts (acid potassium phosphate and sodium chloride), to Solution II, but their presence in the concentrations indicated makes the readings easier and more certain by causing greater opacity of the flocculated lipoids.

Mode of Application.   Into a small test-tube such as ordinarily used for the writer's system of the Wassermann reaction (10 $\times$ 1 cm.) is measured 0.1

c.c. of cerebrospinal fluid, and 1 c.c. of the reagent
is then added. A normal spinal fluid remains perfectly
clear or becomes only faintly opalescent, while a
dense general turbidity is produced in all specimens
containing an increased amount of globulin or albu-
min. In cerebrospinal fluids from bacterial meningitis
the flocculation is dense and copious, followed by
complete or partial sedimentation of the granular
flocculi within about an hour at room temperature.
Specimens from general paresis and tabes dorsalis
give a dense flocculation, somewhat less copious than
those from acute inflammatory conditions, the granular
flocculi settling to the bottom of the tube within a
few hours.

The reaction is rapid, the maximum opacity being
reached within a few minutes; the granulation and
subsequent sedimentation of the flocculi, however,
require a longer time. The reaction takes place at any
temperature, from that of the ice box to that of the
incubator, but room temperature has been found
most satisfactory. If haste is necessary, the reaction
can be read within ten minutes, but a supplementary
reading after the tubes have been left at room tem-
perature for several hours or over night is advisable.
A mild reaction is indicated by a slight general tur-
bidity best recognized in reflected light.

The method has been applied to a large number of
specimens of cerebrospinal fluid, and comparative

observation shows that the test runs parallel with the butyric acid reaction and hence promises to be a useful addition to the diagnostic methods applied to the cerebrospinal fluid.

Quantitative Estimation of Protein Content. The simplest way of estimating the protein content of a given specimen of spinal fluid is by comparing the intensity of the reaction with that obtained with definite quantities of serum proteins. A series of tubes containing graduated quantities of serum is prepared and the reagent added to each. There will be all grades of reaction ranging from total precipitation to slight turbidity, according to the amount of proteins. Table xlvii shows the rapidity and intensity of the reaction with human serum.

As the table shows, the presence of 0.00006 gm. brought about precipitation in one hour. A spinal fluid which gives precipitation with the reagent in one hour must therefore contain approximately 0.00006 gm. per 100 c.c. and one which gives slight turbidity, 0.000024 gm. This method is of course not strictly quantitative. To make more accurate estimation a larger quantity of spinal fluid, such as will give turbidity under normal conditions, must be used, and comparison made by the nephrometric or colorimetric method with a standard containing a known amount of proteins.

TABLE XLVII

TITRATION OF PRECIPITATING POWER OF LIPOIDAL REAGENT

Serum proteins in 0.1 c.c.*	Clear reagent, 1 c.c.			Reagent + cholesterin	
	1 hour	2 hours	24 hours	1 hour	24 hours
0.00024 gm.	ppt.	ppt.	ppt.		
0.00018	ppt.	ppt.	ppt.		
0.00012	ppt.	ppt.	ppt.		
0.00009	ppt.	ppt.	ppt.		
0.00006	ppt.	ppt.	ppt.		
0.000042	Turbid	ppt.	ppt.		
0.00003	Turbid	Turbid	ppt.	All turbid	All precipitated, including control
0.000024	Slight turbidity	Slight turbidity	Turbid		
0.000018	Trace turbidity	Trace turbidity	Turbid		
0.000012	Almost clear	Almost clear	Turbid		
0.000006	Clear	Clear	Clear		
0.	Clear	Clear	Clear		

* Dilutions in 0.4 per cent NaCl of standard containing 0.635 gm. per 100 c.c. as determined by the Kjeldahl method.

NONNE-APELT METHOD

Phase I. 0.3 c.c. of cerebrospinal fluid is mixed with an equal volume of a neutral saturated solution of chemically pure ammonium sulphate in distilled water. A positive reaction (euglobulin) is indicated by precipitation in three minutes.

Phase II. The precipitate is filtered and one drop of 10 per cent acetic acid added to the filtrate. The mixture is then boiled. A positive reaction is indicated by precipitation (serum albumin).

ROSS-JONES METHOD

The method is similar to the Nonne-Apelt, except that the saturated ammonium sulphate solution (0.3 c.c.) is gently poured into a test-tube containing an equal volume of cerebrospinal fluid, just as concentrated nitric acid is employed for the detection of albumin in the urine. A whitish gray ring appears at the junction of the two liquids in case of increased protein content.

PANDY'S CARBOLIC ACID METHOD

Pure carbolic acid is diluted with distilled water in the proportion of 1:6. To one drop of cerebrospinal fluid is added 1 c.c. of the reagent. The formation of a bluish granular precipitate shows the presence of excess globulin.

SULPHOSALICYLIC ACID AND MERCURIC CHLORIDE

This method is recommended for differentiation between tuberculous and other forms of meningitis. One c.c. of a 3 per cent solution of sulphosalicylic acid, mixed with 1 c.c. of cerebrospinal fluid, causes an abundant precipitate, while 1 per cent mercuric chloride in the same proportions causes little precipitation or none at all. In tuberculous meningitis, however, the mercuric chloride precipitate is said to be three times as heavy as that obtained with sulphosalicylic acid.

Sulphosalicylic acid is a powerful precipitant for albumins and globulins and produces precipitation with normal spinal fluids. In the case of fluids with abnormally increased protein content, a very heavy precipitate is obtained when sulphosalicylic acid is mixed with a large quantity (0.6–1 c.c.) of spinal fluid. With 0.1 c.c. of spinal fluid, however, the precipitate obtained with sulphosalicylic acid is less than that obtained with the new lipoidal reagent, notwithstanding the latter reagent causes no precipitation in the case of normal fluids.

Sulphosalicylic acid has also been recommended by Ravaut and Boyer for quantitative estimation of the protein content of cerebrospinal fluid. The estimation is made in the case of the French authors by employing a standard turbidity artificially prepared by suspended silver chloride particles, the density of which is so graduated as to express the amount of the proteins in absolute weight per liter. Denis and Ayer, on the other hand, recommend the use of standardized serum solutions containing 2 mgm. and 3 mgm. protein per 100 c.c. By nephrometric comparison of this standard with a given specimen of cerebrospinal fluid, the protein content of the latter is determined.

LANGE'S COLLOIDAL GOLD TEST

Lange's test for cerebrospinal fluid seems to be based upon the presence in the fluid of two or more

kinds of proteins which induce or inhibit the precipitation of colloidal gold suspension. A stabilized aqueous suspension of colloidal gold is ruby red in color. Its equilibrium is, however, readily disturbed by the introduction of certain substances such as sodium chloride or globulins, and a bluish granular precipitate is formed, but if a trace of albumin has been previously added the precipitation by the salt or globulin is prevented. It is supposed that the particles of colloidal gold acquire a surface film of the albumin emulsion rendering them insusceptible to the action of the ions of the electrolyte which would otherwise bring about precipitation by neutralization of the electrical charges of the particles. A normal spinal fluid contains a trace of both protein fractions, but under various pathological conditions the amounts of both are increased in varying proportions, and the degree of protective or precipitating effect of the spinal fluid may be characteristic for different groups of pathological conditions. No attempt is made to analyze the fluid for the various fractions of protein, but a number of progressively higher dilutions (1:10 to 1:10,240) is tested and the behavior of each noted. The majority of spinal fluids from cases of general paralysis contains a relatively larger quantity of globulin and produce precipitation of the colloidal gold solution in the higher concentrations. Fluids from purulent meningitis, on the other

hand, produce precipitation in the lower concentrations. When these results are represented as curves, they are found to be rather characteristic for certain pathological conditions, and the curves are spoken of as paretic, luetic, meningitic, or normal, according to the zone and degree of precipitation of the colloidal gold suspension.

The nature of the reaction makes necessary the most rigid chemical cleanliness of all glassware used; the slightest trace of protein, acid, or alkali will vitiate the test. The preparation of the main reagent, the colloidal gold suspension, is the most important part of the test.

Preparation of Colloidal Solution of Gold. The following aqueous solutions are prepared: A 1 per cent solution of gold chloride, a 2 per cent solution of potassium carbonate, a 1 per cent solution of formaldehyde, *i.e.*, 1 c.c. of the commercial (40 per cent) solution of the gas in 100 c.c. of water. The water used should be doubly or triply distilled. To 1000 c.c. of the distilled water in a Jena beaker are added 10 c.c. of the gold chloride and 10 c.c. of the potassium carbonate solutions, and the mixture is brought to boiling. Certain workers recommend adding these solutions when the temperature has reached 60–90°C., and removing the flame before the boiling point is reached. Immediately after removing from the flame 10 c.c. of the formaldehyde solution are added and the whole shaken violently. The liquid presently assumes a deep ruby red color. If it is properly made 5 c.c. of it will be completely precipitated by 1.75 c.c. of a 1 per cent sodium chloride solution. In addition it must give characteristic results with known normal, paretic, and luetic spinal fluids. The solution is often alkaline and must be made exactly neutral (pH 7). Titration may be made with phenol red, the technique being the same as that ordinarily used in adjusting the reaction of culture media.

Technique. Twelve test tubes (about $1\frac{1}{16}$ by 6 inches) are required for each specimen of spinal fluid (some workers use ten or eleven only). Into the first

tube is measured 1.8 c.c. of a 0.4 per cent sodium chloride solution, and into each of the remaining 11, 1 c.c. Two-tenths c.c. of the spinal fluid to be tested is then introduced into the first tube, thoroughly mixed with the salt solution, and 1 c.c. of the mixture transferred to the second tube, 1 c.c. from the second to

Cerebral syphilis
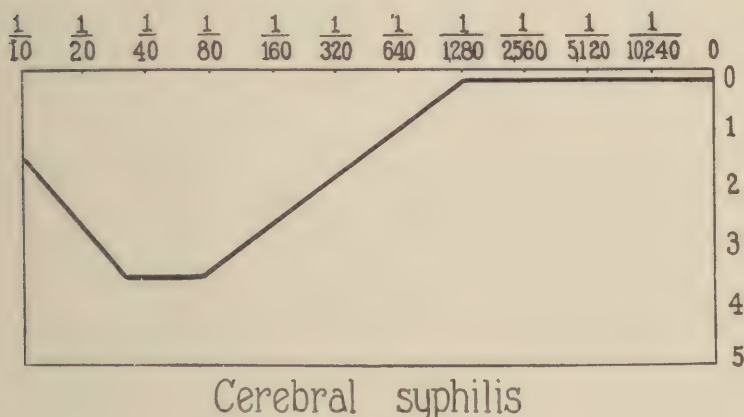
FIG. 13.

the third, and so on up to and including the eleventh tube, from which 1 c.c. is withdrawn and rejected. The twelfth tube is the control; the presence of 1 c.c. of 0.4 per cent NaCl does not precipitate the colloidal gold suspension. The concentration of the spinal fluid thus ranges from a 1:10 dilution in the first tube to a 1:10, 240 in the eleventh. To each of the twelve tubes is now added 5 c.c. of the suspension of colloidal gold, and the tubes are shaken thor-

oughly. Reading of the reaction is made at the end
of 30 minutes at room temperature against a diffuse

General paralysis

Fig. 14.
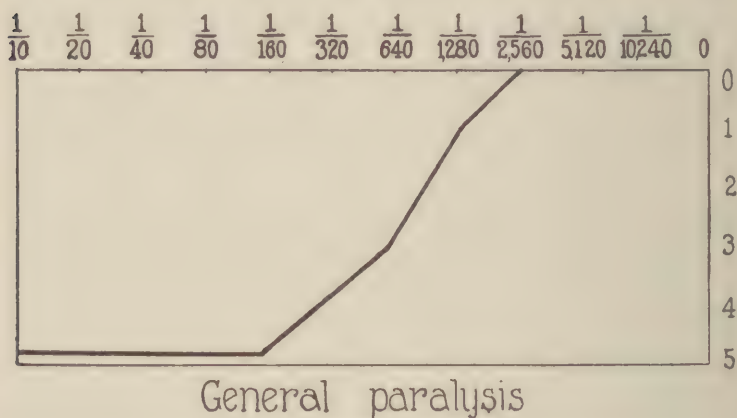
Purulent meningitis
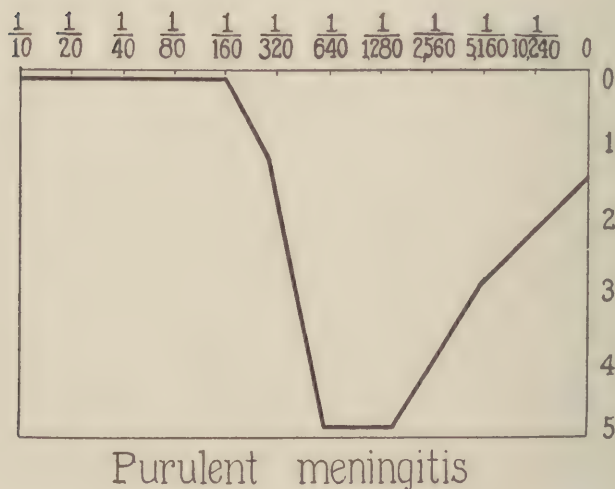
Fig. 15.

white background, the color of the liquid in the
twelfth tube being referred to as the control. The

Tabes.   Purulent meningitis.
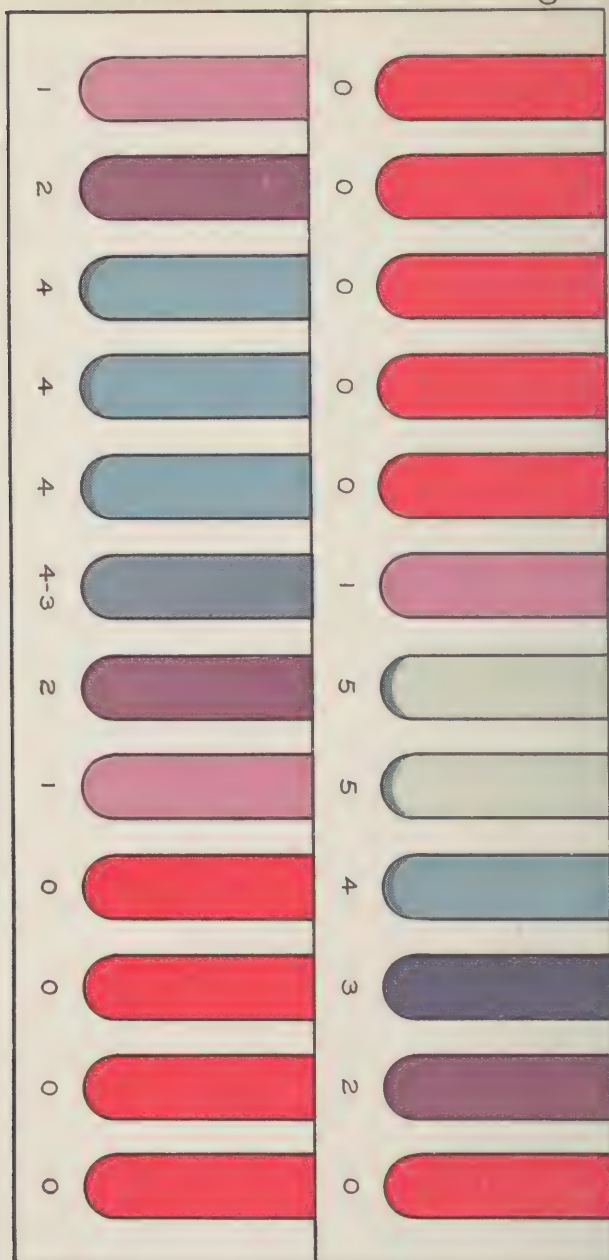
General paralysis. Cerebral syphilis.
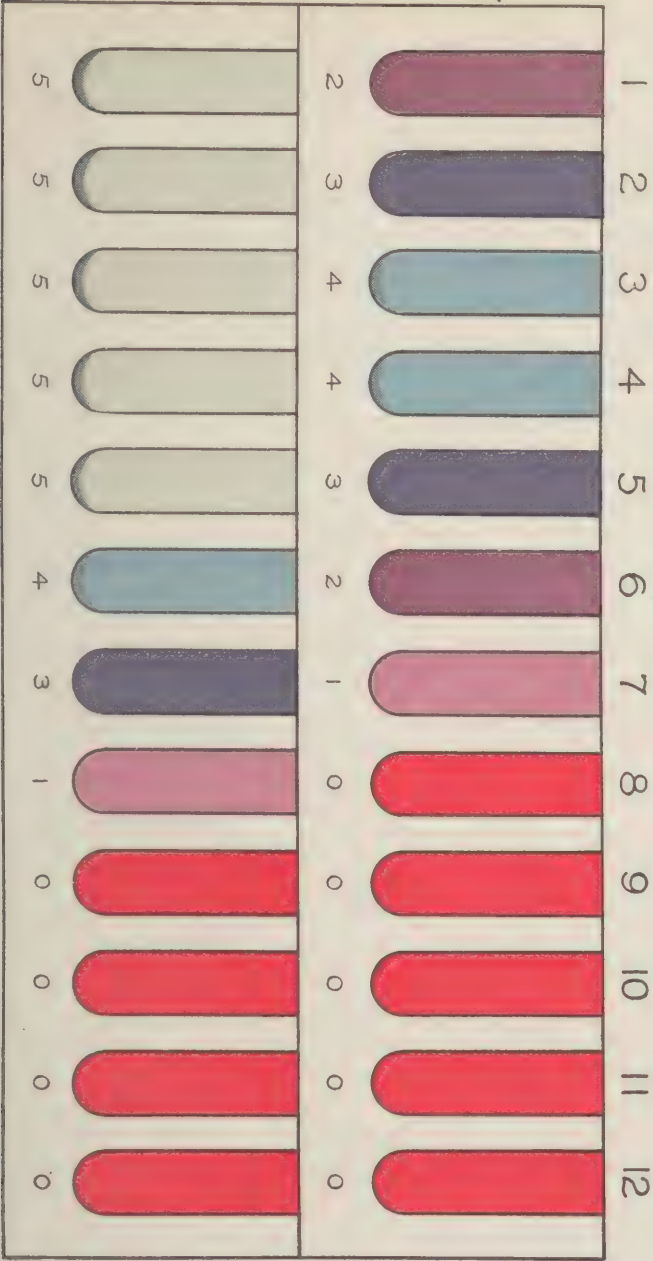
degrees of color reaction are numerically expressed: Red = 0, red-blue = 1, violet-blue = 2, blue = 3, pale blue = 4, colorless = 5. The color values are plotted as the abscissæ against the dilution as ordinates in such a way as to make a continuous curve. Another and final reading is made at the end of 24 hours. The preliminary curve is usually parallel to the final one, but slightly lower in the scale (Figs. 13, 14, 15, and Plate vi).

The colloidal gold test is the most sensitive indicator for pathological changes in the spinal fluid, the butyric acid test approaching it in delicacy. The test does not in all instances differentiate luetic spinal fluid (uremia, neurasthenia, serous meningitis, hemiplegia, and chronic alcoholism may give a curve in the syphilitic zone), but it is valuable in detecting early syphilitic involvement of the central nervous system when used in combination with other diagnostic procedures.

Several modifications of the test have been advocated, especially in the method of preparing the colloidal gold suspension. The addition of 10 drops of a 1 per cent solution of oxalic acid crystals, previous to the addition of the formaldehyde, has been recommended. The importance of neutralizing the solution has been emphasized by a number of workers. Craig recommends as indicator alizarin red, which becomes brownish red when the solution is neutral.

RESULTS OF EXAMINATION OF CEREBROSPINAL FLUID
IN SYPHILIS AND PARASYPHILITIC DISEASES

The results obtained with the butyric acid reaction
will serve to illustrate the value in psychiatry of the
test for increased proteins in the spinal fluid. In col-
laboration with J. W. Moore, the author examined
the cerebrospinal fluid from cases of general paralysis,
tabes dorsalis, dementia præcox, epilepsy, alcoholic
psychosis, senile dementia, and certain other forms
of insanity. The fluids were subjected to the Wasser-
mann test and cell counts made. The cerebrospinal
fluid from several cases of syphilis not showing
involvement of the central nervous system was also
examined.

The specimens of cerebrospinal fluid from the
secondary and tertiary stages of syphilis without
any definite nervous symptoms gave a weak reaction
in the butyric acid test, a normal cell count, and a
negative Wassermann reaction. The cerebrospinal
fluid of a group of cases of hereditary syphilis gave
a positive butyric acid reaction in about 90 per cent
and a positive Wassermann reaction in about 80 per
cent of those examined. On the other hand the cere-
brospinal fluid from cases of cerebral and spinal
syphilis gave a butyric acid reaction in all cases,
showed an increase in number of cells, while the

TABLE XLVIII

RESULTS IN CASES IN WHICH THE DIAGNOSIS WAS REASONABLY CERTAIN

Cases	No. of cases	Butyric acid reaction			Wassermann reaction			Clle count		
		+	−	±	+	−	±	+	−	±
Syphilis:										
Secondary stage....... (without n e r v o u s symptoms)	3	3	0	0	0	3	0	0	3	0
Tertiary stage........ (without n e r v o u s symptoms)	I	I	0	0	0	I	0	0	I	0
Cerebral syphilis......	3	3	0	0	I	I	I	3	0	0
Spinal syphilis........	3	3	0	0	2	I	0	3	0	0
Hereditary syphilis....	10	9	0	I	8	2	0			
General paralysis:										
Cerebral.............	43	37	4	2	32	6	5	39	2	2
Tabetic..............	17	17	0	0	12	3	2	16	I	0
Tabes...............	II	II	0	0	6	4	I	II	0	0
Total..............	91	84	4	3	61	21	9	72	7	2
Psychoses: Arteriosclerotic........	3	I	2	0	I	2	0	I	2	0
Traumatic............	2	0	2	0	0	2	0	0	2	0
Senile...............	I	0	I	0	0	I	0	0	I	0
Epileptic.............	6	0	6	0	0	5	I	0	6	0
Alcoholic.............	7	0	6	I	3	3	I	0	6	I
Manic depressive......	2	0	2	0	I	I	0	0	2	0
Dementia præcox.....	II	I	10	0	I	8	2	I	10	0
Imbecility............	2	0	2	0	0	2	0	0	2	0
Total..............	34	2	31	I	6	24	4	2	31	I

Wassermann reaction was positive in only 50 per cent. Fluids from cases of general paralysis gave positive butyric acid reactions in 90 per cent, increased number of cells in 91 per cent, and positive

TABLE XLIX

ANALYSIS OF THE REACTIONS WITH REGARD TO SYPHILIS

Cases	No. of cases	Butyric acid reaction			Wassermann reaction			Cell count		
		+	−	±	+	−	±	+	−	±
General paralysis and tabes:										
Syphilis admitted......	36	34	1	1	26	8	2	36	0	0
Syphilis denied........	16	13	3	1	10	3	3	11	3	2
Other diseases:										
Syphilis admitted......	1	1	0	0	1	0	0	1	0	0
Syphilis denied........	12	1	11	0	3	8	1	1	11	0

Wassermann reaction in 73 per cent. Fluids from cases of tabes dorsalis gave positive butyric acid reaction and increased cells in all, or 100 per cent, and positive Wassermann reactions in 53 per cent. Finally, fluid from patients suffering from various psychoses without history of syphilis gave positive butyric acid reactions and increased cell count in 2.8 per cent while the Wassermann reaction was positive in 13 per cent.

It is evident that the protein increase runs parallel

with increase of cells in general paralysis and tabes and, in conjunction with cytodiagnosis, the butyric acid reaction is a reliable means of detecting those conditions. The weak reactions to butyric acid of the cerebrospinal fluids from secondary and tertiary syphilis, in which no lesions of the central nervous system are evident, indicate that a protein increase in the fluid is not necessarily associated with an increase and change in the number and kind of cells contained in the fluid. In other words the butyric acid reaction not only indicates the changes in the fluid associated with parasyphilitic and syphilitic lesions of the central nervous system, but in a general syphilitic infection in which the cells are not increased the reaction in the spinal fluid indicates the existence of the pathological condition. Increase in the protein content of the cerebrospinal fluid is usually associated with increase in the number of cells, but less constantly with a positive Wassermann reaction. Furthermore, in rare instances a positive Wassermann reaction was obtained together with a very weak butyric acid reaction, showing that the amount of protein increase has no relation to the amount of syphilitic antibodies present.

Seventeen cases of the series which we examined came to autopsy. Of these, 15 had given positive butyric acid tests, 14 having been diagnosed as cases of general paralysis, and 1 as a case of cere-

16

bral syphilis. Two had given negative tests. The autopsy findings were in complete agreement with the indications of the test.

Rosanoff, Wiseman, and the writer, in a series of investigations conducted at the Kings Park State Hospital, New York, confirmed and extended the observations of Moore and the author, just described. Four hundred and thirteen cases were examined for the Wassermann reaction, protein increase, and cell count. Of this number 252 were available for four simultaneous tests (the Wassermann reaction in serum and in cerebrospinal fluid, butyric acid test, and cell count); in the remaining 161 it was not possible to carry out all of the three tests with the spinal fluid. The Wassermann reaction was done by the writer's system[1] and is designated in the tables as W.-N. In Table L the results obtained with general paralysis are presented. It is interesting to note that the increase of protein and of lymphocytes in the cerebrospinal fluid of these cases is decidedly more constant than the presence of the Wassermann reaction. The latter was more frequently positive in the spinal fluid than in the serum. The four reactions were positive in 70 per cent of the cases examined and all gave positive reactions in at least two different tests. That two or more different tests should give positive reactions is

[1] See Chapter VIII.

TABLE L.

GENERAL PARALYSIS

Variety of test	Serum	Cerebrospinal fluid			No. of cases for each group	Frequency of association of different reactions analysed
	IV.-N.	W.-N.	Butyric acid	Cells		
Grouping of cases according to the frequency and mode of combination of the four different reactions for which they were examined	+ ± + ±	+ + ± ±	+ + + +	+ + + +	24 / 1 / 2 / 4	Four reactions all positive:— 31 = 70 per cent
	− + ± ±	+ + − +	+ + + +	+ ± + −	1 / 1 / 1 / 1	Only three reactions positive:— 7 = 16.4 per cent
	− − −	+ + −	+ ? +	? + +	1 / 1 / 1	Only two reactions positive:— 6 = 13.6 per cent
Number and percentage of positive and negative reactions obtained in corresponding tests	44				44	

Cases with three or more reactions positive:— 38 = 86.4 per cent

Cases with two or more reactions positive:— 44 = 100 per cent

	Serum IV.-N.	W.-N.	Butyric acid	Cells
+	27 = 62%	32 = 72.8%	43 = 97.7%	41 = 93.2%
±	7 = 16.1%	6 = 13.6%	0	1 = 2.3%
−	10 = 22%	6 = 13.6%	1 = 2.3%	2 = 4.5%
+ and ±	78%	86.4%	97.7%	95.5%

quite characteristic of parasyphilitic conditions, for it is very seldom that other forms of psychoses, uncomplicated by syphilis, give plural positive reactions without a definite syphilitic infection. The results obtained with other forms of insanity are

TABLE LI

	Serum	Cerebrospinal fluid			No. of cases in each group	Remarks
	W.-N.	W.-N.	B.	Cells		
Dementia præcox 71 cases	−	−	−	−	45	Syphilis ascertained in 5 cases all reacting positively to the fixation and butyric acid tests. The fixation reaction was present either singly in serum or cerebrospinal fluid or in both. In remaining positive cases syphilis was unascertainable but cannot be excluded. None showed pleocytosis.
	±	−	−	−	4	
	+	−	−	−	9	
	−	+	−	−	2	
	−	±	−	−	3	
	±	−	±	−	2	
	±	±	−	−	1	
	+	−	±	−	4	
	+	+	−	−	1	
Total negative....	50 = 70 %	64 = 88.8 %	65 = 91.5 %	71 = 100 %	71	
Total doubtful ...	7 = 10 %	4 = 6.6 %	6 = 8.5 %	0		
Total positive....	14 = 20 %	3 = 4.6 %	0	0		
Epilepsy 51 cases	−	−	−	−	36	History of syphilis not obtained in this group of cases, but cannot be excluded from the cases giving positive reactions for the fixation and butyric acid tests. None showed pleocytosis.
	±	−	−	−	2	
	−	±	−	−	1	
	+	−	−	−	8	
	−	−	+	−	1	
	±	−	+	−	1	
	+	+	−	−	2	
Total negative....	38 = 74 %	48 = 94 %	49 = 96 %	51 = 100 %	51	
Total doubtful....	3 = 6 %	1 = 2 %	0	0		
Total positive	10 = 20 %	2 = 4 %	2 = 4 %	0		

TABLE LI—*Continued*

	Serum	Cerebrospinal fluid			No. of cases in each group	Remarks
	W.-N.	W.-N.	B.	Cells		
Senile dementia, 9 cases	−	−	−	−	7	Syphilis probable in cases giving positive reactions for the fixation and butyric acid tests, but difficult to establish with certainty. None showed pleocytosis.
	±	−	−	−	1	
	−	+	+	−	1	
Total negative....	8 = 88.9 %	8 = 88.9 %	8 = 88.9 %	9 = 100 %	9	
Total doubtful....	1 = 11.1 %	0	0	0		
Total positive.....	0	1	1	0		
Manic depressive insanity, 6 cases	−	−	−	−	3	History of syphilis not ascertainable, but probable in cases giving positive Wassermann. None showed pleocytosis.
	±	−	−	−	2	
	+	+	−	−	1	
Total negative	3	5	6	6	6	
Total doubtful.......	2	0	0	0		
Total positive.......	1	1	0	0		
Alcoholic psychosis, 4 cases	±	−	−	−	1	History of syphilis not ascertained in all cases giving positive reactions with the fixation or butyric acid tests. None showed pleocytosis.
	±	−	±	−	1	
	+	−	+	−	1	
	−	+	±	−	1	
Total negative	1	3	1	4	4	
Total doubtful......	2	0	2	0		
Total positive.......	1	1	1	0		

recorded in Table LI. There are a number of cases giving solitary positive Wassermann reaction, but scarcely any that gave positive cytodiagnosis. Again, we find that the butyric acid test was positive in some cases with syphilitic histories and that the Wassermann reaction in the serum was also positive in these cases.

TABLE LI—*Continued*

	Serum	Cerebrospinal fluid			No. of cases in each	Remarks
	W.-N.	W.-N.	B.	Cells	group	
Polyneuritic psychosis, 7 cases	−	−	−	−	6	History of syphilis not obtained in the case reacting positively to the fixation test in serum. The fixation test in spinal fluid, butyric acid test and pleocytosis negative in all.
	+	−	−	−	1	
Total negative	6	7	7	7	7	
Total positive	1	0	0	0		
Involution melancholia, 7 cases	−	−	−	−	6	History of syphilis not ascertained in the positive case. All reacted negatively to to the fixation test in spinal fluid, butyric acid test and cytodiagnosis.
	+	−	−	−	1	
Total negative.......	6	7	7	7	7	
Total positive........	1	0	0	0		
Paranoiac condition, 4 cases	−	−	−	−	3	History of syphilis not ascertained.
	+	−	−	−	1	
Total negative	3	4	4	4	4	
Total positive	1	0	0	0		
Imbecility............. 4 cases	−	−	−	−	2	History of syphilis not ascertainable.
	+	−	−	−	2	
Total negative.......	2	4	4	4	4	
Total positive........	2	0	0	0		

Stern studied the protein increase with the butyric acid test in a series of psychiatric conditions and obtained the following results: General paresis: 5⁻; 52 positive. Tabes dorsalis, 3; all positive. Cerebrospinal syphilis, 1: positive. Forty-eight nonsyphilitic individuals were studied as controls. The controls include the following forms of psychosis: Dementia præcox, manic depressive insanity, melancholia, epilepsy, senility; toxic nephritic, alcoholic, Korsakoff's

and postparalytic psychosis, imbecility, idio-imbecility, and puerperal psychosis. Of these the reaction was positive only in two cases of profound uremia and one of postparalytic dementia. The reaction was positive with the spinal fluids of eight cadavers.

TABLE LI—*Continued*

	Serum	Cerebrospinal fluid			No. of cases in each group	Remarks
	W.-N.	W.-N.	B.	Cells		
Infantile cerebral palsy, 4 cases	—	—	—	—	4	Syphilis not ascertained.
Arteriosclerotic dementia, 7 cases	—	—	—	—	7	Syphilis not ascertained.
Brain tumor........ 1 case	—	—	—	—	1	Syphilis not ascertained.
Traumatic psychosis, 1 case	—	—	—	—	1	Syphilis not ascertained.
Unclassified....... 32 cases	—	—	—	—	18	Syphilis ascertained in 5 cases reacting positively either to the fixation or to the butyric acid test or to both. In other positive cases it was not ascertained but cannot be excluded. None showed pleocytosis.
	±	—	—	—	4	
	+	—	—	—	3	
	—	—	±	—	2	
	—	—	+	—	2	
	+	—	+	—	1	
	±	+	—	—	1	
	±	—	±	—	1	

McCampbell and Rowland made a comparative study of the Wassermann reaction, butyric acid test, Ross-Jones test, and cell counts on 46 cases of general paralysis, 5 cases of dementia præcox, 2 cases of paranoia, 1 case of melancholia, 2 cases of manic depressive insanity, 3 cases of secondary lues and 2 cases of tertiary lues, including 2 normal individuals.

Their results are largely confirmatory of those already described, except that a much higher percentage of positive Wassermann reactions was obtained by them with the blood sera from parasyphilitic cases. Their findings were exactly the same as those of others with regard to the cerebrospinal fluid, namely, the percentage of positive reactions in the paretics was 85.7 per cent for the Wassermann reaction and 95.6 per cent for the butyric acid test and pleocytosis. The percentage of positive reactions with the Ross-Jones test was slightly lower than with the butyric acid test. The results obtained with the cerebrospinal fluids were the same in the 10 non-parasyphilitic psychiatric cases in every test and were uniformly negative. In all 3 secondary cases of syphilis the butyric acid and Ross-Jones tests were positive, but negative for the Wassermann reaction and pleocytosis. One of the 2 tertiary cases was positive for the butyric acid and the Ross-Jones reactions and the other was doubtful for the butyric acid and negative for the Ross-Jones test. All five specimens of spinal fluid gave negative Wassermann tests and normal cell counts. On the other hand, the sera of these syphilitic cases all gave positive Wassermann reactions as would be expected. Finally specimens of the cerebrospinal fluid and serum from 2 normal individuals reacted negatively to all tests.

PROTEIN INCREASE IN DISEASES OTHER THAN SYPHILIS

In all acute and subacute inflammations of the meninges there is an increase of proteins in the spinal fluid. There is likely to be no difficulty whatever in the separation of this class of cases, as has already been mentioned (p. 223). The only instance of inflammation which might be confused with a syph-

TABLE LII

THE PROTEIN INCREASE IN THE CEREBROSPINAL FLUID IN DISEASES OTHER THAN SYPHILIS

Cases	No. of cases	Butyric acid reaction			Wassermann reaction		
		+	−	±	+	−	±
Diseases of the meninges:							
Epidemic cerebrospinal meningitis	14	14	0	0	0	14	0
Pneumococcus meningitis	6	6	0	0	0	6	0
Influenza bacillus meningitis	1	1	0	0	0	1	0
Tuberculous meningitis	30	30	0	0	0	30	0
Hydrocephalus externus	2	2	0	0	1	1	0
	53	53	0	0	1	52	0
Diseases without meningeal involvement:							
Typhoid fever	1	0	1	0	0	1	
Pneumonia	4	0	4	0	0	4	0
Pulmonary tuberculosis	1	0	1	0	0	1	0
Enterocolitis	2	0	2	0	0	2	0
Rachitis	1	0	1	0	0	1	0
Uremia	2	0	2	0	0	2	0
Septicemia	1	0	1	0	0	1	0
Miscellaneous without nervous involvement	12	0	11	1	0	10	2
	24	0	23	1	0	22	2

ilitic affection is tuberculous meningitis, in which the fluid is clear and contains an excessive number of mononuclear cells. But this affection is readily distinguished not only by its clinical history, but also by the presence of the tubercle bacilli.

A positive reaction with any test for increased proteins will be given by the cerebrospinal fluid from tuberculous meningitis but the Wassermann test will be negative. Hence the two tests, together with the clinical history, will provide means of diagnosis even before microscopic detection of the tubercle bacilli or animal inoculations have given evidence of the disease.

CHAPTER XVI

METHODS FOR THE DETECTION AND STUDY OF TREPONEMA PALLIDUM

Treponema pallidum is a delicate spiral organism (Figs. 16–19) which varies in length from 6 to 14 microns; the majority are 8 to 12 microns long. In experimentally infected rabbits forms as long as 24 microns are occasionally found, but in general the species is more uniform in length than other treponemata. The body is cylindrical. The width is about 0.25 micron, though occasionally forms as thick as 0.3 micron have been observed. The spiral depth is 0.8 to 1 micron, the spiral amplitude about 1 micron; an organism of average length has 9 to 10 spirals. One or more undulations may also be present.

The characteristic of the genus Treponema is the regularity and depth of the spirals, which in quiescent forms appear rigid. During active movement, however, they may be alternately stretched and relaxed while the organism constantly rotates on its long axis. The body often bends near the middle, and very active specimens sometimes form a ring by bringing the two ends together. Both ends are pointed, and an extremely delicate filament at
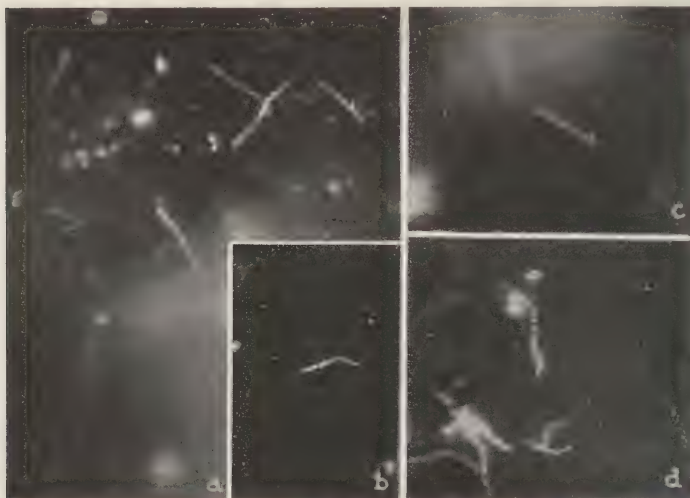
FIG. 16. *Treponema pallidum*. Darkfield views. Magnification × 1000. (*a*) From experimental syphilitic orchitis in the rabbit. (*b* and *c*) From human chancre. (*d*) From a pure culture.

FIG. 17. *Treponema pallidum*. Film preparation stained by Dr. A. C. Coles, showing terminal filaments. From prints kindly furnished by Dr. Coles.

one or both ends is occasionally visible by darkfield illumination and can be brought out by special
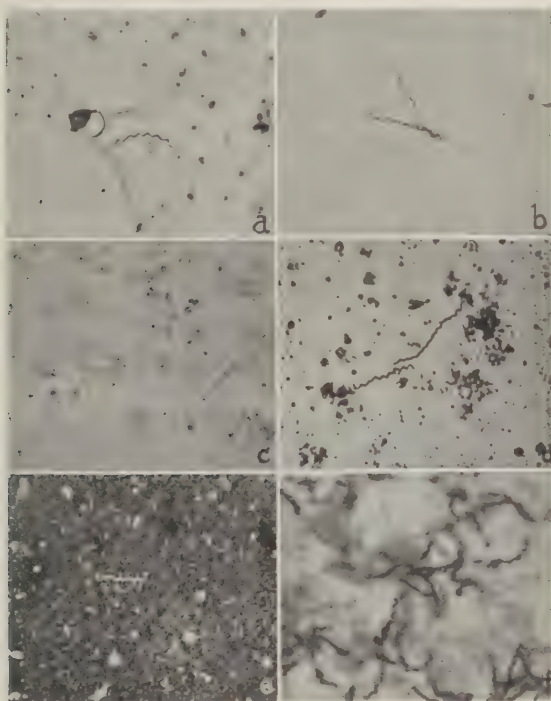
Fig. 18. *Treponema pallidum.* Magnification × 1000. (*a*) Film preparation stained by a modification of Loeffler's method for flagella (p. 297). (*b*) Film preparation from human chancre, fixed in buffered formalin and stained with carbol fuchsin (p. 297). (*c*) Film preparation, fixed in methyl alcohol and stained with Giemsa solution (p. 294). (*d*) Film preparation, stained by Fontana's method (p. 287). (*e*) Film preparation, stained by Burri's India ink method (p. 285). (*f*) Section of liver from a congenitally syphilitic fetus. Levaditi's method of silver impregnation (p. 299), magnification × 1280.

staining such as that of Coles (Fig. 17). In stained preparations forms with stretched spirals are often seen.
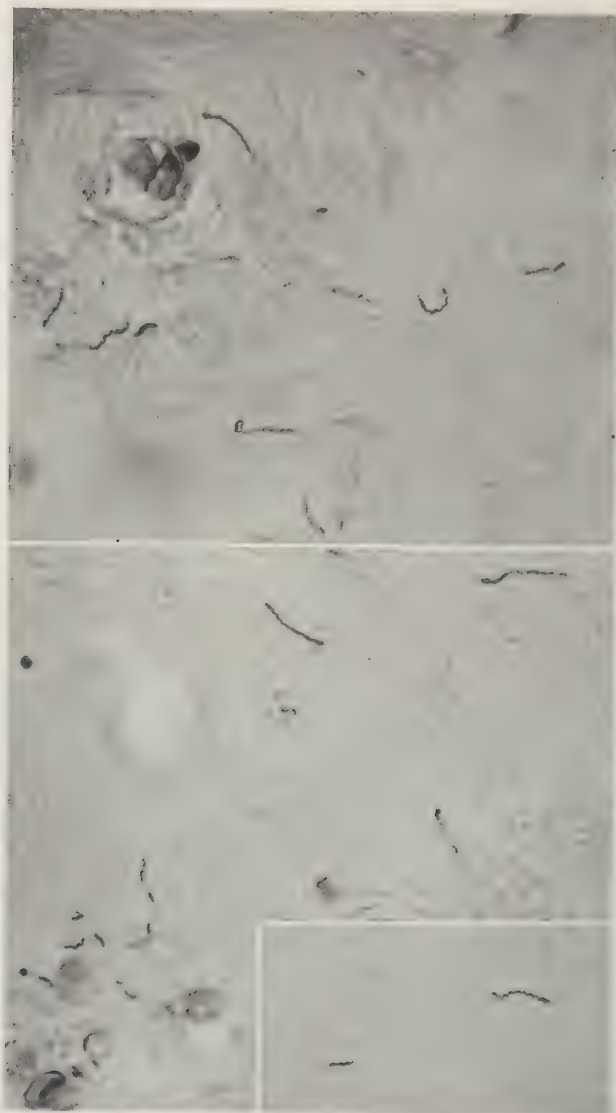
FIG. 19. *Treponema pallidum* in the brain from general paralysis. Noguchi's method of silver impregnation for nerve tissues (p. 300). Magnification × 1000.

The organism is very graceful in its movements, which are never violent. This characteristic, together with the regularity of spirals, and the whiteness of the light refracted by the organism under the dark-field, are important distinguishing features. There are, however, several organisms which may be confused with *Treponema pallidum*, two of which are morphologically indistinguishable from it.

Fig. 20. *Treponema cuniculi* from spontaneous venereal infection in the rabbit. Darkfield views. Magnification × 1000.

Treponema cuniculi (Figs. 20 21), found in the spontaneous venereal infection of rabbits, is morphologically very similar to *Treponema pallidum;* it is perhaps slightly coarser and longer. The lesions produced by *T. cuniculi* are papulosquamous and often ulcerating; the writer has not been able to produce in rabbits with this organism a typical orchitis or keratitis such as is incited by *Treponema pallidum.* The Wassermann test, in 23 rabbits infected with this organism, was found by the writer to be

uniformly negative, while in rabbits infected with *Treponema pallidum* it is usually positive.
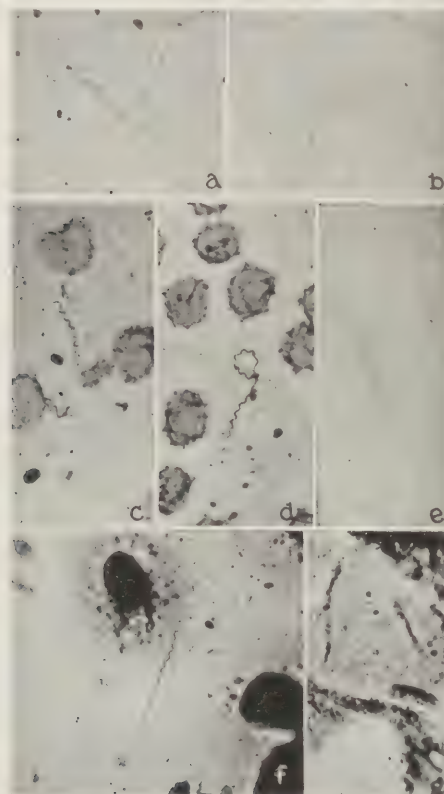
FIG. 21. *Treponema cuniculi* from spontaneous venereal infection of rabbits. Magnification × 1000. (*a*) Film preparation, fixed in osmic acid and absolute alcohol (p. 293) and stained with Giemsa solution (p. 294). (*b*) Same, fixed in methyl alcohol and stained with Giemsa solution (p. 294). (*c* and *d*) Same, stained by Fontana's method (p. 287). (*e*) Same, mordant gentian violet (p. 297). (*f*) Same, fixed in buffered formalin and stained with carbol fuchsin (p. 297). (*g*) Section of testicle. Levaditi's silver impregnation method (p. 299).

Treponema pertenue (Fig. 22) of yaws is morphologically indistinguishable from *T. pallidum*.

Treponema pallidum, *T. cuniculi*, and *T. pertenue* are similarly affected by arsenobenzol, that is, the drug brings about disappearance of the organisms and healing of the lesions.

Treponema microdentium (Fig. 23), an organism
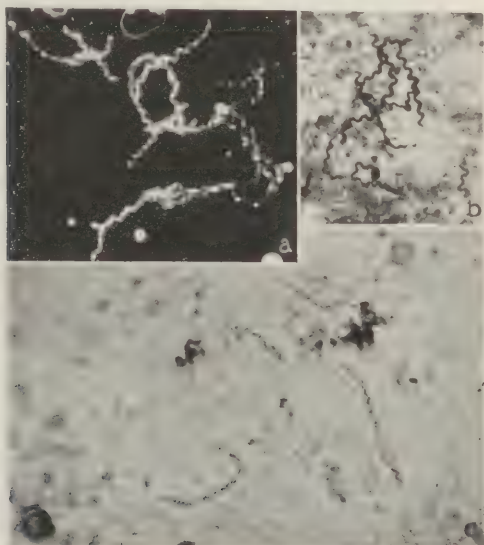
FIG. 22. *Treponema pertenue* (yaws). Magnification × 1000. (a) Pure culture. (b) Section from lesion, stained by Levaditi's silver impregnation method (from Bull. No. 1, U. S. Army, Washington, 1913). (c) Film preparation, Giemsa stain. After Nichols.

found in the normal mouth, is also very similar in morphology to *Treponema pallidum*. In perfectly fresh preparations, however, its movements are slightly different, the terminal portions being more active than the middle and vibrating constantly. This terminal vibration is not observed in *pallidum*, *cuniculi*, or *pertenue*. The body of *microdentium*

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is seldom bent at the middle, while the *pallidum-cuniculi-pertenue* group constantly shows this characteristic in fresh preparations. The spirals of *microdentium* are more closely set than those of the
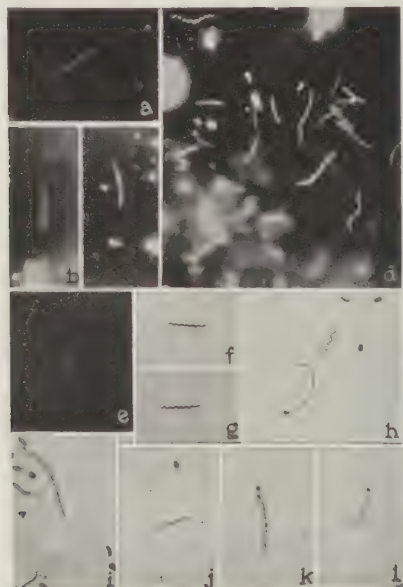
FIG. 23. *Treponema microdentium* (human mouth). Magnification × 1000. (*a*, *b*, *c*) Darkfield views of preparations from mouth. (*d*) Darkfield view of pure culture. (*e*) Film preparation from mouth, stained by Benians' method (p. 286). (*f* and *g*) Same, stained by Fontana's method (p. 287). (*h*) Same, showing a leptospira-like organism. (*i*, *j*, *k*, *l*) Various specimens, stained by the mordant gentian violet method (p. 297).

pallidum. The length of this organism varies from 4 to 8 micron; the width is about 0.2 to 0.25 micron.

Treponema macrodentium (Fig. 24) and *Treponema mucosum* (Fig. 25), both inhabitants of the mouth, likewise are similar in appearance to the *pallidum*

group. *Treponema macrodentium* varies from 5 to 12 micron in length and from 0.35 to 0.6 micron in width, that is, it is a thicker organism than *pallidum;*

Fig. 24. *Treponema macrodentium* (human mouth). Magnification × 1000. (a) Darkfield view of preparation from mouth. (b) Film preparation, stained by Benians' method (p. 286). (c) Same, stained by Burri's method (p. 285). (d) Darkfield view of a pure culture. (e and f) Film preparations, stained by Fontana's method (p. 287). (g) Same, stained by mordant gentian violet method (p. 297).

furthermore, the coils are fewer in number and not so deep. *Macrodentium* and *mucosum,* like *micro-*

dentium, show greater activity of the two or three coils at each end, and the ends are often slightly bent.

Treponema calligyrum (Fig. 26), found in smegma on the normal genitalia, appears like *pallidum* on a somewhat larger scale. Although usually about the same length as *pallidum*, that is, 6 to 14 micron, *calligyrum* is thicker (0.35 to 0.4 micron),
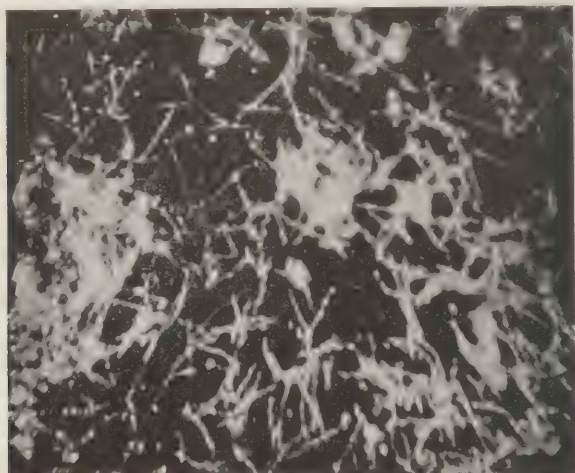
Fɪɢ. 25. Treponema mucosum (Pyorrhea alveolaris). Darkfield view of a pure culture. Magnification × 1000.

and even the very short specimens (5 to 6 micron) occasionally found are not difficult to distinguish from the *pallidum*.

Treponema genitalis[1] (Fig. 27), also found in

[1] This organism was described by the writer (*J. Exper. Med.*, 1918, xxvii, 667) under the name of *Treponema minutum*, but since it was found later that Dobell had already used the name (1912) to designate an organism found in the stomach of the toad, the species name has been changed to *genitalis*. Since this variety is the most numerous of the spirochaetae on the normal genitalia, the term is doubly satisfactory.

smegma, is very much like *microdentium*, except that the curves are somewhat deeper and decidedly closer; this organism is considerably shorter (7 to 10 micron)
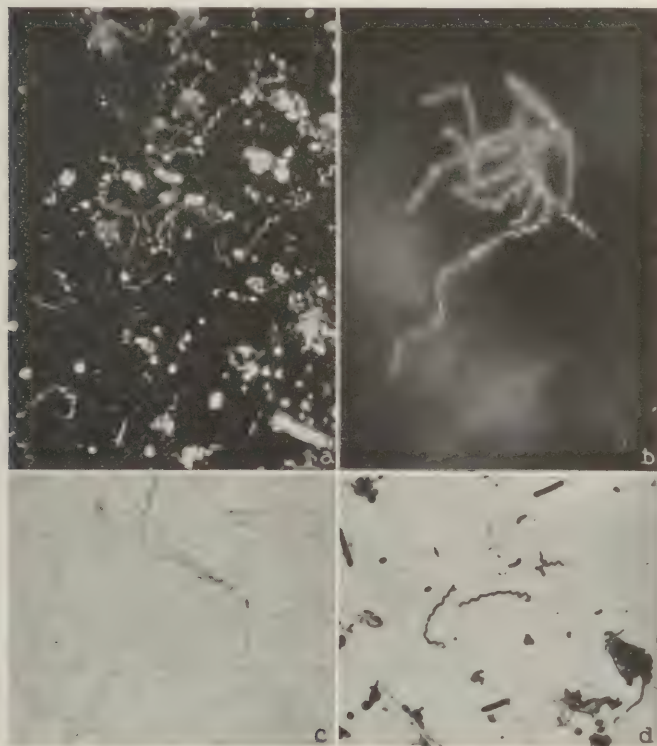
FIG. 26. Treponema calligyrum (normal genitalia). Magnification × 1000. (*a*) Darkfield view of a pure culture (solid medium). (*b*) Dark-field view of a pure culture (fluid medium). (*c*) Film preparation of pure culture, stained by the mordant gentian violet method p. (297). (*d*) Film preparation from smegma, stained by Fontana's method (p. 287).

than *pallidum*, and very short forms (3 micron) are frequently found. The width is about the same as that of *pallidum*, i.e., 0.25 to 0.3 micron.

Treponema refringens (Fig. 28), while easily differentiated from *T. pallidum*, is important because of the frequency with which it is encountered in the
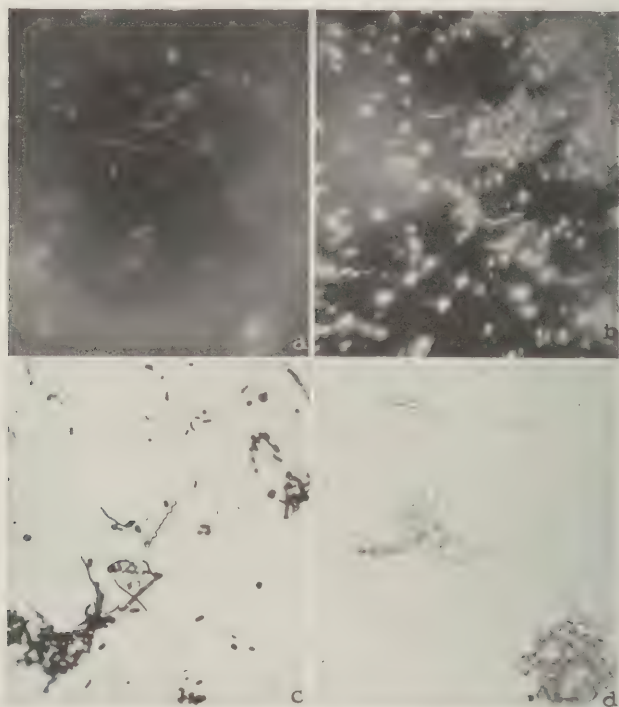
FIG. 27. *Treponema genitalis* (normal genitalia). Magnification × 1000. (a) Darkfield view of preparation from smegma. (b) Darkfield view of pure culture. (c) Film preparation from smegma, stained by Fontana's method (p. 287). (d) Film preparation of pure culture, stained by the mordant gentian violet method (p. 297).

scrapings from genital lesions. It is interesting because described by Schaudinn and Hoffmann in their first publication on *T. pallidum*.

The mouth and smegma treponemata, when fixed in methyl alcohol, stain more easily and deeply with Giemsa than the *pallidum-cuniculi-pertenue* group.

Fig. 28. *Treponema refringens* (normal genitalia). Magnification × 1000. (*a*) Darkfield view of preparation from smegma. (*b*) Film preparation from smegma, stained by Fontana's method (p. 287). (*c*) Young, short forms in pure culture. Darkfield view. (*d*) Darkfield view of pure culture. (*e*) Film preparation of pure culture, stained by Fontana's method.

DARKGROUND ILLUMINATION

The darkfield microscope is exceedingly important for the study of *Treponema pallidum*, because it

brings out clearly the natural morphological features and movements of the organism and details of structure which stained preparations often fail to show.

The principle of darkfield illumination is a simple

FIG. 29. Single surface (paraboloid) condenser constructed by Wenham in 1856. After Jentzsch.

one. An object invisible to our eyes when suspended in a diffuse light may be rendered visible when the observer is protected from receiving diffuse light and is placed in such a position as to receive only those rays which are either reflected from the surface of the object or are refracted by the object (self-luminosity). The situation is analogous to that familiar to everyone in the dancing of minute luminous particles of dust in a stream of bright sunshine flashed through a small opening into a dark room or in a current of artificial light projected through a dark room—the darker the room the more distinctly visible the dancing particles become. The principle was applied in microscopy by Wenham, who in 1856 constructed a paraboloid reflecting (mirror) surface condenser (Fig. 29). The method was much improved by J. W. Stephenson in 1879, who introduced his "catoptric immersion illuminator,"

which is a spherical reflecting surface darkground illuminator similar to that later brought into use by Reichert, von Ignatowsky, Siedentopf, and Jentzsch.

Apparatus and Manipulation

The conditions requisite to self-luminosity under the microscope are that the object have a refractive index different from that of the medium in which it is
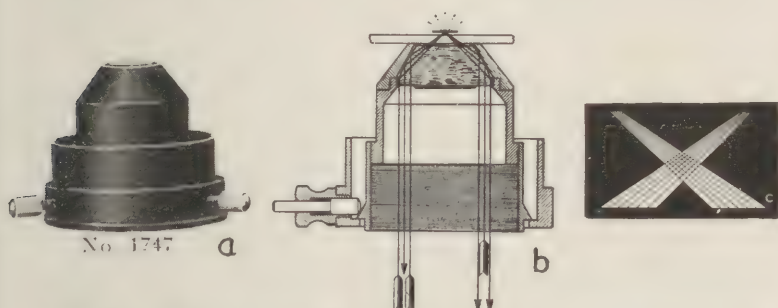
FIG. 30. (a) Single surface (paraboloid) condenser, which is centered by means of the lateral screws. (b) Diagram (cross section) showing the course of the rays of light passing up through the zone outside the central stop to the reflecting surface on the walls of the condenser, from which they are reflected and converge at the point where the object lies. (c) The path of the reflected rays of light.

suspended or embedded, and that the rays of light which illuminate it fall outside the objective of the microscope, only the rays refracted or reflected by the object itself being permitted to reach the observer. Under these conditions the object appears light, the ground, dark. In other words, the darkfield microscope must have an illuminator which throws the light obliquely on the object and at such an angle

that none of the direct rays enter the objective. To accomplish this result, an opaque disc is inserted beneath the condenser which cuts off all rays except those coming from a narrow peripheral zone, and these latter are caused to converge, usually by means of a reflecting surface, at the point where the object lies.

There are several types of optical construction which bring about luminous images on a dark ground:
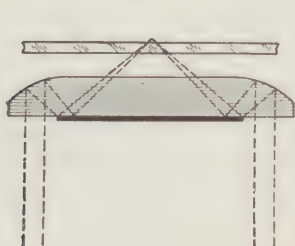
FIG. 31. Single surface condenser (Reichert). After Chamot.
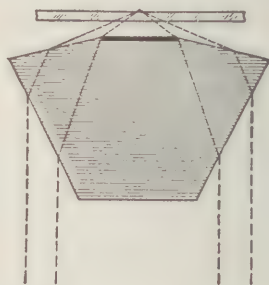
FIG. 32. Refracting condenser of Nachet et fils. After Chamot.

The paraboloid reflecting surface condenser introduced by Wenham in 1856 (Fig. 29) and improved by Stephenson (1879). This is the darkfield condenser now marketed by Zeiss, and by Bausch and Lomb (Fig. 30). Another form of the single surface reflecting condenser is shown in Fig. 31.

The bispherical reflecting surface condenser, which is of three distinct forms. The first (Fig. 33) was designed by von Ignatowsky in 1907 and taken up by Leitz, the second (Figs. 34-35), designed by Jentzsch

somewhat later, is the one now recommended by Leitz. The third (Fig. 36) was constructed by Siedentopf in 1910 and placed on the market by Zeiss under
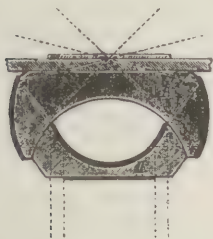
FIG. 33.

FIG. 34.

FIG. 33. Diagram (cross section) of the bispherical reflecting condenser of v. Ignatowsky. After Jentzsch. The heavy lines indicate the reflecting surfaces, the dotted lines the course of the rays.

FIG. 34. Bispherical surface reflecting condenser of the Jentzsch type (Leitz), which is centered by means of the lateral screws
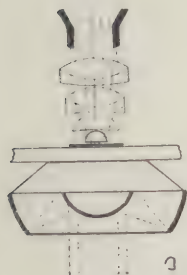
FIG. 35.
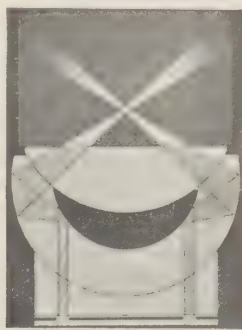
FIG. 36.

FIG. 35. (a) Diagram (cross section) of the bispherical reflecting condenser of the Jentzsch type in conjunction with oil immersion objective. The heavy lines indicate the reflecting surfaces, the dotted lines the course of the rays of light. (b) Shows how the rays converge to a small point.

FIG. 36. Diagram (cross section) of Siedentopf's bispherical surface (cardioid) reflecting condenser.

the name of the cardioid condenser. The darkfield condenser sold by the Spencer Lens Co. is also of the bispherical type.

An achromatic (Fig. 37), or an aplanatic achromatic condenser, the latter designed by Metz
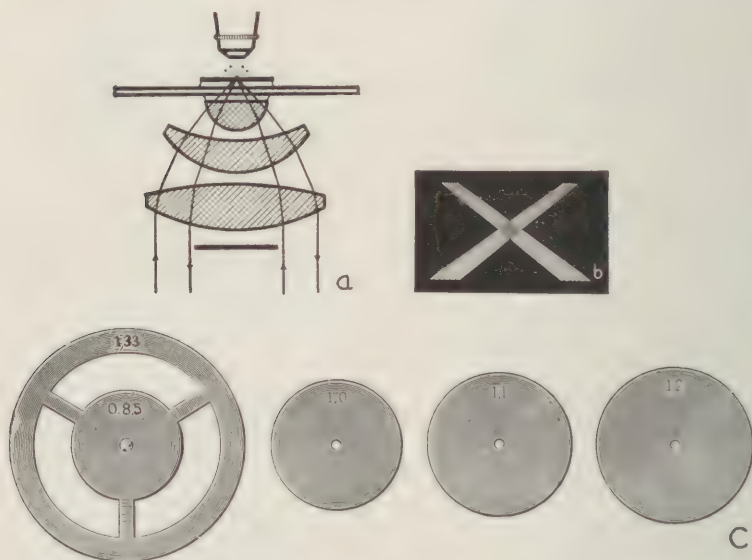
FIG. 37. (a) Diagram (cross section) of the three lens achromatic (Abbe) condenser with central stop inserted beneath to bring about darkfield illumination (dry system). The rays passing up through the zone outside the central stop are refracted by the lenses and converge as shown in (b). After Barnard. (c) The central stop.

(Fig. 38), with numerical aperture 1.35 to 1.4, to which a stop (Fig. 37c) may be applied in the substage stop carrier. The size of the stop is dependent upon the numerical aperture of the objective, hence a set of stops of different sizes is desirable.

The Condenser. The refracting condensers (achromatic or aplanatic) are not suitable for darkfield work with the highest powers (i.e., higher than 4 mm.).

According to Coles, however, a very satisfactory dark ground illumination may be obtained with an achromatic condenser (Fig. 37) used dry with a central stop (Fig. 37c) which can be rotated in and out of position at will, so that one can pass from dark ground to ordinary illumination without removing the eye from the microscope. (This advantage is now obtainable with the new combination condensers—see pp. 271-3.) Coles uses as light source an ordinary paraffin lamp with a ½ inch wick and small bull's eye. The objective recommended by him is the 8 mm. apochromatic Zeiss, and the ocular a compensating 6 for general search, the operator passing if need be to ocular 8, 12, or 18. The tube length for which the objective is corrected, that is, 170 mm., is suitable for fresh coverglass preparations, but in the case of stained preparations the tube length is increased to 200 mm. to compensate for the absence of the coverglass. Coles states that in such a system the inconvenience of using oil is entirely dispensed with. Water is probably used to make the contact between condenser and slide. An aplanatic achromatic condenser (Fig. 38) may be substituted for the achromatic with as good, or better, results.
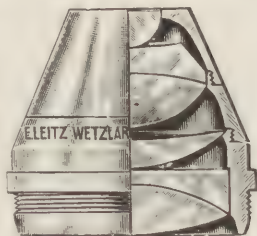
Fig. 38. The aplanatic achromatic condenser of C. Metz which may be made to produce darkfield illumination (dry system) by the introduction of a substage central stop like that shown in Fig. 37c.

All of the reflecting condensers, however, are capable of giving good service when properly adjusted, and both types (paraboloid and bispherical) are widely employed for the study of spirochetes.

Whatever the illuminating system used, it is essential that full central illumination be obtained. The operator must not be satisfied with a partial or side illumination, which, although it may reveal the general shape of the organisms, fails to bring out

details of structure and also fails to differentiate certain elements derived from tissue or culture medium which are often mistaken for organisms, but which do not bear scrutiny under full central illumination. Considerable practice may be required before satisfactory results are obtained. The horizontal position of the condenser must be accurately centered to the optical axis of the microscope, and its vertical position adjusted in accordance with the thickness of the object slide. It is important that the bundle of rays from the illuminant should completely cover the mirror.

It is easy to see why the darkground condenser or illuminator must be adjustable as to its vertical as well as its horizontal position. The reflected or refracted rays come to a focus at a certain point (Figs. 35b, 37b), and at this point the illumination is full or all-sided. There is only one focal point which gives sufficient illumination to an object lying in the same horizontal plane, and unless the optic axis of the objective is passing through this point no luminosity of the object can be obtained.

The newest Leitz darkfield condenser is so constructed that it no longer requires centering, the diameter of the condenser being increased so that it just fits into the central opening of the microscope stage; it is only necessary to center the light by means of the mirror and to adjust the vertical position

of the condenser. The older Leitz darkfield condenser is held by two adjusting screws and a spring within a collar (Fig. 34), and its horizontal position must be adjusted to the optic axis of the microscope. The raising or lowering of the condenser is achieved as usual by rack-work.
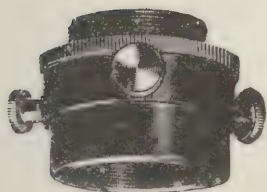
FIG. 39. Nosepiece centering device.
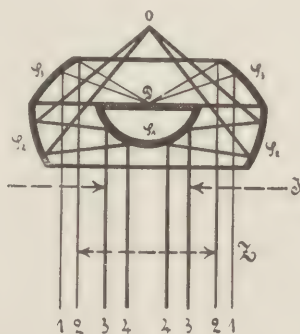
FIG. 40. A diagrammatic sketch of the Leitz bright- and darkfield condenser, showing the course of the rays of light (see text).

In the case of the Zeiss darkfield system, the centering device (Fig. 39) is attached to the nosepiece, just above the objective, the horizontal position of the condenser being fixed.

Combination Bright and Darkfield Condensers. The Leitz combination reflecting condenser is shown in Figs. 40 to 44. When the iris diaphragm is closed far enough to exclude the rays indicated in the diagram (Fig. 40) as 1-2, and the central stop swung out to admit rays 3-4, the effect is the same as that of the

ordinary darkground condenser. Fig. 42 shows the condenser in this position. When, however, the iris diaphragm is open (Fig. 43) and the central stop in
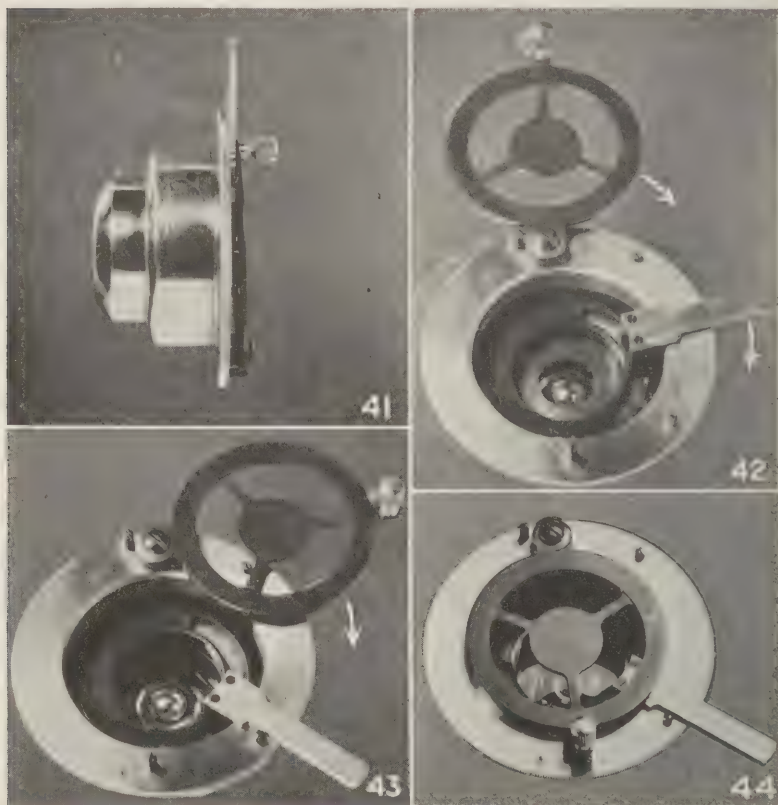
FIGS. 41–44. Bright and darkfield condenser of Leitz. Fig. 42 shows the central stop swung out and iris diaphragm partly closed for use as a dark-field condenser. With the iris diaphragm open (Fig. 43) and the central stop swung in (Fig. 44) it becomes a brightfield condenser.

place (Fig. 44), the effect produced, with an objective of numerical aperture 0.80 to 0.95, is that of bright-field illumination.

The "improved paraboloid condenser" or "Wechselkondensor" of Zeiss (Fig. 45) likewise permits transition from bright to darkfield illumination, and is also adjustable to slides of different thickness.

Fig. 45. The improved paraboloid condenser (Wechselkondensor) of Zeiss for light and dark ground illumination. One lever makes the adjustment to thickness of slide, the other regulates the position of the central stop and of the iris diaphragm.

The condenser is provided with two levers, one of which makes the adjustment to the thickness of the slide, the other swings the central stop in for darkfield or out for brightfield illumination and also regulates the iris diaphragm for use in brightfield work. The maximum numerical aperture of the objective which can be used is 0.85.
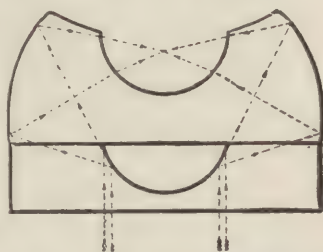
Fig. 46. Diagram of the hanging drop condenser (after Jentzsch).

By means of these condensers, the darkfield apparatus may be set up in a permanent way and made to serve also the purpose of brightfield illumination. If it is impossible to have more than one microscope, such an arrangement is preferable to frequent changing of condenser and

illuminating system, but otherwise it is more satisfactory to have two separate apparatus, because the darkfield illuminating system is not well suited to brightfield work.

Hanging Drop Condensers. For studying particles suspended in a gas or liquid a so-called hanging drop darkfield condenser has been devised by Jentzsch for Leitz and also by Oelze for Zeiss, in which the second reflecting surface is so constructed as to bring about convergence of the rays in the center of a semi-spherical hollow space ground out in the upper part of the condenser (Fig. 46), where the object to be examined is placed. This type of condenser has not been perfected for use in bacteriology.

The Source of Light. A much more intense light than that used for ordinary microscopy is required for darkfield work. There are several devices for the purpose, but for routine diagnostic work a nitrogen bulb with small incandescent tungsten coil with a condensing lens (Bausch and Lomb) is
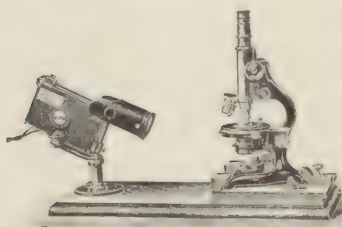
Fig. 47. "Lilliput" portable arc lamp.

satisfactory. For prolonged research, and especially in field work, a portable arc lamp is preferable. Such arc lamps (Fig. 47) are made by Leitz, Bausch and Lomb, and Zeiss. A very satisfactory type of illuminant for ordinary darkfield work is the Pointolite, a tungsten arc in a gas-filled bulb (100 candle power), which gives an intense light of small area. This lamp was originally made in England, but can be obtained, with suitable housing, from the Bausch and Lomb

Optical Co. Direct current only can be used. For photographic work a much longer exposure (2 minutes, e.g., instead of 10 seconds) is required with the Pointolite than with an arc lamp. The Spencer Lens Company furnishes a powerful (500 candlepower) electric lamp which gives excellent results in both dark- and brightfield work.

The Objective. In order that none of the light reflected by the illuminator (but only that reflected by the object) can enter the objective, the numerical aperture of the latter should not exceed 0.95 mm. Reduction of the N.A. of the ordinary oil immersion objective is accomplished by placing a funnel stop in the back of the objective (Fig. 48). The stop is either screwed or just dropped in. It is readily procured from the makers at small cost. An objective $1\frac{1}{12}$ oil immersion with ocular 3 or 4,

FIG. 48. The funnel stop which must be placed in any objective of aperture higher than 0.95 for use with the darkfield condenser.

or a 2 mm. apochromatic objective with compensating ocular 6 or 8, is usually recommended for spirochetal work. One may use also apochromatic dry objectives of 8 mm., 4 mm., or 3 mm., in combination respectively with compensating oculars 18, 12, or 8. With all of these the funnel stop must be used.

For use with the improved paraboloid condenser Zeiss has a 3 mm. "X" apochromatic objective of

numerical aperture 0.85 which, with a compensating ocular 12 or 18, gives excellent results both with dark and with brightfield illumination. This objective may also be used satisfactorily with the darkfield and combination condensers of other firms.

The Leitz 3.45 mm. oil immersion objective, designated 1/7 a, has a numerical aperture of 0.95 and may be used in conjunction with the combination condenser for dark- and brightfield work. Not being apochromatic, it is less expensive than the other medium aperture objectives. A compensating ocular 12 or 18 is required for the higher magnifications.

The Bausch and Lomb Optical Company makes a high power (1.9 mm.) apochromatic objective of medium numerical aperture (0.80) for dark- and brightfield work. With this an ocular 4 is used.

Slides and Coverglasses. The thickness of slides is specified by the makers of the darkfield illuminators; the limits of error are said not to exceed 0.1 mm.; 1 to 1.2 mm. is a satisfactory thickness. A slide as thick as 1.5 to 1.7 mm. can be used with the paraboloid condensers. The coverglasses should be from 0.15 to 0.18 mm. thick. These specifications are very rigid for a dry lens, but less so for an oil immersion lens; in the latter case the only requirement is that the slide must not be so thick that the objective cannot focus through it. When using a dry objective, one must

take precautions to obtain the clearest possible image by adjusting the tube length.

In any darkfield system removal of the air between the darkfield condenser and the *under surface of the slide* is essential whether a dry objective or an oil immersion is being used. This is done by placing a few drops of cedar oil between them. In darkfield work with an oil immersion objective cedar oil is placed between the condenser and the slide on the one hand and between the coverglass and the objective on the other. Water may often be substituted for oil in making the contact between the condenser and the slide. With some types of condenser, however, the water cannot be kept in place and a more coherent liquid must be used.

Setting Up the Darkfield Apparatus. To set up a microscope with a substage darkfield condenser the light source is placed at such a distance from the mirror of the microscope that the cone of concentrated light from the illuminant will cover the whole surface of the plain (*not* the concave) mirror.

The centering of the darkfield condenser is easily done with a low power: for example, objective 3, ocular 2. Two concentric rings are usually ruled on the condenser (Fig. 49). These are brought into the center of the field by means of the screws in the collar of the condenser (Fig. 34). In the case of some of the newer condensers, with a diameter exactly

that of the opening in the stage, no centering is necessary; it is only necessary to center the light by means of the mirror.

The material to be examined[1] is put in the center of a thin slide (1 mm.) by means of a Pasteur capillary pipette, the amount being such as to spread as thinly as possible when covered. The coverglass should be so
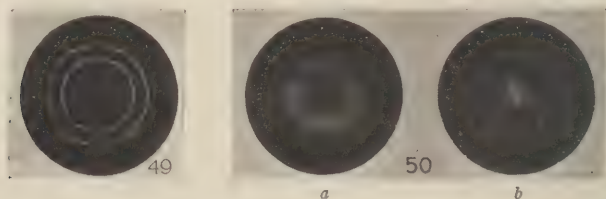
FIG. 49. Concentric rings ruled on a darkfield condenser to facilitate centering.

FIG. 50. (a) The peripheral illumination of the low power field when the condenser is too low. (b) The bright central illumination when the condenser is in the correct position.

thin as to bend somewhat when held by the edges between the thumb and index finger (No. 0) and preferably of the dimensions of 22 × 22 mm. Before putting the preparation down on the stage, a large drop of cedar oil is put on the under side of the slide and a smaller drop on the coverglass. The preparation is now ready to be placed on the stage. Raise the condenser[2] until it is in contact with the inverted

[1] The deposits found at the base of the teeth in the normal mouth contain a large number of spirochetes of several types and when mixed with a little distilled water or saline solution make excellent preparations for the purpose of adjusting the darkfield microscope.

[2] Some condensers are not adjustable as to vertical position. The "Wechselkondensor" of Zeiss, for example, is vertically fixed but has a lever for making the adjustment to the thickness of the slide.

drop of oil and farther until the space between con-
denser and slide is completely filled, no air bubbles
being present. Lower the coarse adjustment until a
bright spot (Fig. 50b) or a ring of light (Fig. 50a) is
seen, then raise the condenser until the spot is made
as small and bright as possible and if it is not in the
center of the field adjust the mirror to bring it to
the center.

The object is now ready for examination under a
higher magnification. An oil immersion objective,
with funnel stop inserted (Fig. 48) and a high power
ocular are put in place. The objective is lowered by
the coarse adjustment until the oil is reached and still
farther until some image is seen. If the field appears
vaguely light in the center, near the center, or even
toward the periphery, the mirror must be adjusted
to center the light; if by going farther one finds a more
definite spot, but nothing sharp, the darkfield con-
denser is still a little too low, that is, the focus is at a
point below where the object lies. By gradually
raising the substage condenser and raising the
objective by the fine adjustment simultaneously the
maximum illumination is reached and a sharply
defined image appears. If the field appears greenish
yellow in the center, with bright yellow circle, the
focus of the darkground illuminator is *above* the
object. In this case the condenser and objective are
simultaneously lowered until a sharp, well illuminated

object comes to view. Full, central illumination is thus secured.

With the concave mirror one gets fairly sharp illumination, but the images appear very thin, and the interior structure of the organism cannot be recognized. By using the peripheral zone of illumination with the plain mirror one gets a similar, side-illuminated picture. Differential diagnosis based upon observation with a lateral or partial illumination is unreliable (pp. 269–270).

Once centered, the darkfield substage condenser is best not disturbed, since much time is required for readjustment. For this reason, it is desirable to have a microscope for darkfield work alone. Many workers also advise not changing the adjustment between the illuminant, the bull's eye condenser, and the darkfield substage condenser, but when one becomes accustomed to a darkfield system readjustment is easily made.

Cleaning of Used Slides and Coverglasses. The best way to clean the slides and coverglasses used for darkfield work is to put the preparation after examination in a mixture of xylene and chloroform (1000 parts of xylene to 10 of chloroform) in a jar with air tight lid. Xylene dissolves the oil, and both substances are germicidal.[1] Benzene may be used instead

[1] It has been found that several hours are required for the cleaning fluid to penetrate between slide and coverglass, hence in the case of infectious material it is safer to separate them under the surface of the fluid with a knife to permit rapid sterilization.

of xylene. The slides and coverglasses come apart easily and can be recovered without the haze so often resulting from the use of lysol. The cleaning mixture may be used repeatedly.

Indications and Material for Darkfield Examination

Delicate organisms such as *Treponema pallidum* can be brought to view with more certainty by the so-called darkfield illumination than by any other method for direct detection at present available (see Fig. 16). They may be examined by this means in the fresh condition, unstained, or, as recently shown by Erich Hoffmann, even after being fixed and stained, in film preparations and sections. The advantage of darkfield examination of fresh material is the simplicity and facility by which the organisms can be seen in their natural form and characteristic movement; even if they have lost their motility their characteristic forms are easily recognized by dark-field illumination. Specimens preserved in 10 per cent buffered formalin solution (Tilden), as described below, have also been found very satisfactory for darkfield examination as well as for staining.

Hoffmann's method (p. 284) makes possible observations on organisms in preserved specimens which have been prepared many years and have faded. It shows, for example, many more *Treponema pallidum* than the ordinary microscope in sections of the brain of

general paretics stained by the method of silver impregnation, because certain specimens which are obscured by tissue elements can be differentiated by their characteristic refraction of light under the darkfield illumination.

Technique for the Collection of Material

Initial Lesions. The surface is cleansed of superficial débris with a piece of gauze moistened with saline and the lesion squeezed slightly to express a little of the tissue fluid. The fluid is drawn into a capillary pipette (Pasteur type), placed on a clean slide (1 mm. in thickness), covered with a thin coverglass (No. 0), and it is ready for darkfield examination.

If no *pallidum* is found by this means, a cataract knife or small curette may be used to scrape the superficial layer of the chancre. The scraping is mixed with a drop of saline and examined. If the finding is still negative, a small piece of the chancre may be excised by means of a small sharp pair of curved scissors. This is easily done by a single snipping. The removed tissue is ground in a mortar with a small amount of saline and the resulting suspension examined in the way described above. The latter method often yields a positive result when other means have failed, and a negative report should not be made unless this technique has also been tried.

Miss Tilden has found that emulsions made in 10 per cent buffered formalin in place of saline keep the organisms morphologically unmodified for a very long period (at least one year), hence specimens of this sort may, if necessary, be forwarded to distant laboratories for examination.

Mouth Lesions. After superficial cleansing, the lesion is scraped with a small sharp curette, and the scrapings, suspended in saline, used for examination.

Skin Lesions. Papules may be scraped and squeezed or dry-cupped. Macules may be blistered and the fluid expressed by pressure.

Lymph Glands. Puncture with a syringe needle and inject a few drops of sterile saline solution into the gland, then aspirate back with simultaneous pressure of the gland. The material in the calibre of the needle is forced out on a slide.

Cerebrospinal Fluid. Occasionally *Treponema pallidum* has been demonstrated in the cerebrospinal fluid in neurosyphilis.

In experimental syphilis in animals the demonstration of the *pallidum* is accomplished by means similar to those already described.

Darkfield Examination of Fixed and Stained Preparations

This method of search for *Treponema pallidum* had been tried occasionally since the introduction of the darkfield microscope into general use in 1906, but

the recent work of Hoffmann has brought it into practical use. He describes his method as "Leucht-bildmethode" because of the luminous image produced by the process of darkfield illumination. The luminosity of the stained objects has a character-istic color for each stain used. A Giemsa-stained *pallidum* (pink), for example, appears somewhat greenish, a Levaditi-stained *pallidum* (dark brown) yellowish green, a tubercle bacillus (red by carbol fuchsin) bright green, and a blue staining organism brown. These facts suggest a phenomenon of fluores-cence emanating from the stained organism, but careful study seems to indicate that by darkground illumination the complement color of the stain is refracted and perceived by the observer. Hoffmann's improvement over the early procedure of Arning (1908) is due to his use of a tannin solution for differentiation of Giemsa preparations.[1] By this method the *pallidum* appears pale whitish, or yellowish white, while other forms are coarser and look bright green. For the examination of silver stained sections, Hoffmann introduced the use of a ground glass to be placed before the mirror; this accentuates the difference in the refractive power of

[1] The unfixed smear is covered with a solution containing 1 to 2 drops of 1 per cent K_2CO_3 and 20 to 25 drops of Giemsa solution in 10 c. c. of water, heated to steaming, the fluid decanted, and the process repeated 3 or 4 times; differentiation is then made by treating the preparation for 1 to 2 minutes in a 25 per cent aqueous solution of tannin.

the *pallidum* and the tissue fibers and renders the recognition of the former much easier than otherwise. As stated previously, the writer has confirmed the findings described by Hoffmann.

STAINING OF FILM PREPARATIONS

A. Negative Image Methods

A number of staining methods are based on the principle of using a pigment which will stain the background uniformly and leave the spirochetes unstained; the spiral form of the organism is seen as a blank on a stained background. The finer the particles covering the ground the sharper the negative image of the spirochete.

1. *Burri's India Ink Method* (Figs. 18e, 24c). One drop of the fluid to be examined is mixed on a slide with one drop of India ink, and the material spread in the same way as a blood film. It is important not to repeat the spreading on the same slide. The preparation may be examined as soon as the film is dry. The spirochetes appear white on a black or brownish black granular background. This was the first of the negative image methods and has been improved upon by later workers.

2. *Harrison's Collargol Method.* Harrison substitutes collargol (1 part to 19 parts distilled water) for the India ink. The powder is put into a black bottle, water added, the bottle stoppered and shaken at intervals. A drop is mixed on a slide with a drop

of the material to be examined, and the mixture spread like a blood film. The spirochetes appear as delicate spirals on a homogeneous reddish brown field.

3. *Benians' Method* (Figs. 23*e*, 24*b*). A drop of a 2 per cent aqueous solution of Congo red (filtered) is mixed with a drop of the material to be examined and the mixture spread. After drying in the air the slide is immersed in acid alcohol (1 per cent HCl in absolute alcohol), whereby the background is converted into a semi-opaque bluish purple. The preparation remains unchanged for several days.

B. Silver Impregnation in Film Preparations

One of the most reliable means of demonstrating spirochetes in dried films is that recommended by Fontana. His original method has been modified by later investigators, but the technique is based on the principle first used by him and gives excellent results.

1. *Fontana's Original Method* (1912). (Figs. 23*f*, *g*; 24*e*, *f*; 26*d*; 28*e*.) The technique consists in the application of a mordant and subsequent reduction of an alkalinized silver nitrate solution, and two solutions are used, (1) a 5 per cent aqueous solution of tannin, and (2) a 5 per cent aqueous solution of silver nitrate, to which ammonia is added to render it slightly alkaline. The concentration of ammonia added should be such that the first drop will produce a precipitate; more ammonia is added, drop by drop, until the precipitate is dissolved, then a drop of 5 per cent silver nitrate solution is put in, and the liquid becomes slightly opalescent. This solution can be preserved for many months.

The material to be examined is diluted with one drop of distilled water, and a film made on the slide, air-dried, and fixed by passing over a flame. A few drops of the mordant solution is poured on and the slide held over the flame until steam rises (about 20 seconds). The slide is washed for 30 seconds in running water, a few drops of the ammoniac silver nitrate solution poured on, and the preparation held over the flame as before (20 to 30

seconds, or until the desired intensity of coloration is obtained), then washed and dried. The total time required is about 70 to 80 seconds. The spirochetes appear deep yellow or brown.

2. *Fontana's Improved Method* (1913). (Figs. 18*d*; 21*c*, *d*; 27*c*). Instead of fixing by heat, Ruge's fluid[1] is poured over the slide and renewed several times in the course of a minute (it is more convenient, when a number of slides are being stained at once, to fill a Coplin staining jar with Ruge's fluid and immerse the slides for 1 to 5 minutes). The mordant solution (5 per cent tannin) is made up in 1 per cent phenol water for the purpose of preservation and perhaps for better mordanting. A plain silver nitrate solution (0.25 per cent instead of 5 per cent) replaces the ammoniac silver nitrate solution, and alkalinization is carried out at the time of use. The three solutions are:

Solution I (Ruge's fluid)	Glacial acetic acid................	1 c. c.
	Formalin........................	20 c. c.
	Distilled water....................	100 c. c.
Solution II (mordant)	Tannin	5 grams
	Phenol...........................	1 gram
	Distilled water....................	100 c. c.
Solution III	Silver nitrate	1 gram
	Distilled water....................	400 c. c.

The films are dried in the air and fixed in Solution 1 for 1 to 5 minutes, then thoroughly washed in running tap water. Solution 11 is poured over the film and warmed until vapor arises, left for 30 seconds,

[1] Although this name appears in some textbooks as Huge, the correct spelling is Ruge.

then washed in running water for 15 to 30 seconds. Without drying, the silver nitrate solution, Solution III, to which ammonia has been added with a capillary pipette until slight turbidity results, is applied. (If excess of ammonia is introduced, the fluid becomes clear again and cannot be used—a trace of ammonia is all that is required.) The preparation is heated until steam arises, left 30 seconds longer, then washed. If permanent preparations are desired, they must be mounted in xylene balsam; cedar oil causes fading of the spirochetes. The jet-black treponemata stand out prominently on the clear yellowish background and appear much thicker than when stained with aniline dyes.

3. *Tribondeau's Modification of Fontana's Method* (1912). Tribondeau introduced some improvements of the original method by substitution of Ruge's fluid for fixation and by adding a preservative (camphor) to the mordant. The advantages of Tribondeau's method have, however, been incorporated into Fontana's second method, just described. Three solutions are required, (1) Ruge's fluid, (2) tannin solution (tannin, alcoholic or ethereal, 1 gram; distilled water, 20 c.c., heated to dissolve the tannin; a small piece of camphor to prevent mould contamination; the solution is kept in a well-stoppered bottle), (3) ammoniac silver nitrate solution as in Fontana's original method. Films are made as usual and air-dried, then fixed in Solution 1, which is poured on and off once or twice, and a fresh quantity left to act for 1 to 5 minutes. Films made with secretions from syphilitic ulcers should be washed afterwards with absolute alcohol, those containing fats also with alcohol, ether, and again with alcohol. After fixation the films are covered with the tannin mordant (Solution II) and held over the flame until vapor rises (not boiled); the mordant is left for 30 seconds longer, the slide washed in tap (30 seconds) and then in distilled water. The silver solution is first allowed to act for a few seconds in the cold, the first poured off and fresh quantity added and heated until vapor rises. The film, which is of a maroon color, is washed, dried and mounted in neutral glycerol if a permanent preparation is desired.

4. *Ravaut and Ponselle's Largine Method.* The
Austrian organic silver preparation known as largine
is employed in place of silver nitrate. An albu-
minate of silver gives equally satisfactory results.

5. *Warthin and Starry's Silver Agar Method for
Coverglass Films.* Prepare smears on No. 1 cover-
glasses, and dry thoroughly in the air.

Place in absolute alcohol for 3 to 5 minutes, then
wash in distilled water. Treatment with concentrated
hydrogen peroxide for 5 to 20 minutes will clear the
background; the preparation must be thoroughly
washed with distilled water after this treatment.

Rinse the coverglass in 2 per cent silver nitrate,
cover the smear side with another perfectly clean
coverglass, and place the adherent pair of coverglasses
in 2 per cent silver nitrate solution in the incubator
for 1 to 2 hours.

Remove the coverglasses from the silver solution,
and separate them. Place smear side up in the reduc-
tion fluid:

2 per cent silver nitrate solution...............................	3 c.c.
Warm glycerol...	5 c.c.
Warm 10 per cent aqueous gelatine...........................	5 c.c.
Warm 1½ per cent agar suspension[1]........................	5 c.c.
5 per cent aqueous hydroquinone solution......................	2 c.c.

[1] The agar suspension is prepared as follows: 1.5 gr. of agar, repeatedly
washed with distilled water, is put into 100 c.c. distilled water, then
brought to the boiling point, when it goes into solution. It is poured into a
clean bottle and is constantly shaken or stirred to prevent uniform solidi-
fication. A thick semifluid suspension results which is kept on the top of a
paraffin oven, stoppered with a cork. At the time of use the agar suspension
is added to the other ingredients and the hydroquinone put in last.

After reduction is complete, remove the coverglass and rinse in 5 per cent sodium thiosulphate solution.

Rinse in distilled water, dehydrate with absolute alcohol, clear in xylene, and mount in balsam.

Warthin and Starry believe not only that their technique gives better differentiation than other methods of silver impregnation in smears, but also that by it *Treponema pallidum* can be differentiated from other spirochetes of similar morphology (e.g., mouth spirochetes) with more certainty than by the darkfield microscope.

If the darkfield microscope is properly used, however, no staining technique can be superior in the differentiation of species, because the changes in morphology—due to the degree of impregnation, the condition of the organisms at the time of fixation, etc.—brought about by the processes of fixing and staining are considerable. Neither the natural width of the organisms nor the nature of the curves can be determined as certainly in a stained preparation—no matter how well stained—as in fresh preparations observed under the darkfield microscope. Furthermore, the advantage of observing the characteristic motility of *Treponema pallidum*, as compared with the movements of the smegma and mouth spirochetes is obvious (pp. 257–260).

6. *Perrin's Method for Fixing Impregnated Films.* To prevent fading of smears stained by the Fontana

or other silver impregnation methods, Perrin recommends treating the stained smear with a commercial fixing and toning solution, whereby the yellow background disappears, the organisms become dark gray to bluish black, and the preparation is rendered permanent. If it is desired that the organisms appear actually black, the following toning and fixing bath is used:

Ammoniumsulfocyanide.	6.25	gm.
Tartaric (or citric) acid	0.5	gm.
NaCl	1.25	gm.
Distilled water	250	c.c.
Gold chloride solution (1 per cent)	6.25	c.c.

C. Aniline Dye Staining Methods

Unlike most bacteria, the spirochetes do not readily take up the average basic aniline dye. The coarser groups stain more easily than the finer varieties. The most difficult to stain are those belonging to the Treponema and Leptospira groups; Cristispira and Spirochaeta (*plicatilis* type) stain rather easily with most dyes; the *recurrentis* group occupies an intermediary position with respect to stainability. By means of suitable fixation, however, any of the spirochetes, including *Treponema pallidum*, can be made to stain distinctly with either basic fuchsin or gentian violet (pp. 297–298).

Almost all of the highly specialized stains owe their use to recent developments in medical protozoology

and hematology, and particularly to the discovery of *Treponema pallidum*, which, though its recognition is so important at an early stage of the infection, seemed to be almost unstainable with the bacterial stains known at the time of its discovery. Even the most difficultly staining varieties of spirochetes, however, can be stained with the polychromatic aniline dyes, of which Giemsa's, Wright's, Leishman's, and Laveran's preparations are best known and most reliable.

Schaudinn introduced the azure eosin compound for staining the *pallidum*, the original technique of Schaudinn and Hoffmann being as follows:

1. Fix coverglass films in absolute alcohol for 10 minutes.

2. Stain for 24 hours, film side down, in the following solution (mixed freshly at time of use):

    Giemsa's eosin-solution........................ 12 parts
      (2.5 c.c. of 1 per cent eosin solution in 500 c.c.
      water)
    Azur I.. 3 parts
      (1 gm. in 1000 c.c. water)
    Azur II....................................... 3 parts
      (0.8 gr. in 1000 c.c. water)

3. Rinse the preparation in distilled water for a few seconds, dry in the air, and mount in cedar oil.

This method was improved and simplified by Giemsa, whose formula is now being followed by various firms, the most reliable preparation being that of Grübler.

1. *Giemsa Stain* (Figs. 18c; 21a; 22c)

The solution can be used in two different ways: One is to apply it several times in rapid succession and

with slight steaming over the flame; the other is to leave the film in a dilute solution for a longer period at room temperature. The maximum staining may be attained in two hours. Giemsa-stained preparations are always mounted in cedar oil, because balsam decolorizes them.

Fixation of Films for Giemsa's Stain. Giemsa recommends fixation of the film, after drying in air, in chemically pure, neutral, methyl alcohol for 30 minutes. Exposure of the freshly spread film to osmic acid vapor for 30 to 60 seconds and after-fixation, before the film begins to dry, in absolute alcohol is a common procedure in modern cytological studies and has been applied to *Treponema pallidum* and other spirochetes by Hoffmann and others. Exposure to the fumes of a mixture of chloroform and iodine and after-hardening in absolute alcohol (Plymmer's method) has been found to give satisfactory fixation (Coles). On the other hand, Coles has shown that for bringing out the finest structural details it is best not to fix the film. He makes smears as thin as practicable and allows them to dry in air. When they are entirely dry a few drops of distilled water, previously made slightly alkaline, are poured over the surface and allowed to remain for about 30 seconds. The films are then stained in Giemsa solution for 3 to 4 hours.

Rapid Giemsa Staining. The film, first dried in the air, may be fixed with methyl alcohol for 5 to 10

minutes or over the flame (Schereschewsky). Cover the film surface with 4 to 6 drops of concentrated Giemsa (stock) solution, add 10 to 12 drops of distilled water (neutral or slightly alkaline), mix, and warm over the flame until steam begins to rise. Hold outside the flame for a minute, pour off. Repeat this process at least three times, leaving the mixture for five minutes the last time. Rinse in distilled water and dry.

Wright's stain[1] is used in a similar way and gives excellent results.

The rapid method has been recommended by Shmamine, Schereschewsky, and several others, the principle and results being practically the same. Hoffmann's technique for rapid staining differs somewhat. The unfixed film is covered with a mixture, containing in 10 c. c. of distilled water, 1 to 2 drops of 1 per cent potassium carbonate solution and 20 to 25 drops of Giemsa solution. The film is warmed over the flame until it steams, and the procedure is repeated 3 to 4 times. The film is differentiated in 50 to 60 per cent tannin solution, washed, and dried.

Slow Giemsa Staining. This is the procedure ordinarily meant in connection with Giemsa's stain. A more dilute solution of the stain is used and no heat applied. The concentration recommended by Giemsa

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[1] A reliable preparation of Wright's stain is obtainable from Hynson Westcott and Dunning, Baltimore, Md.

is 1 drop of the stock solution to each cubic centi-
meter of distilled water. If several slides are to be
stained at the same time, it is convenient to use a
Coplin staining jar and add 40 c. c. of distilled water
to 2 c. c. of the stock solution of the stain. The dis-
tilled water may be rendered neutral or slightly
alkaline by the addition of a drop of potassium
carbonate solution (1 per cent).

The minimum time for staining is 1 hour at room
temperature; the results are better after 2 hours,
but longer staining than this gives no further improve-
ment, and in some instances, particularly when
slides have been fixed in methyl alcohol too long (an
hour or longer) the stain is less well differentiated
after 24 hours, the background showing more intense
reddish precipitate than after an hour's standing.

In a properly stained Giemsa preparation (fixed
in methyl alcohol) the erythrocytes are pinkish yellow,
the nuclei of leucocytes reddish purple, protoplasm
and granules are well differentiated according to
their nature, and *Treponema pallidum* is pinkish red.
In a film fixed in alcohol and ether the color of the
erythrocytes is pink, while in osmic acid preparations
it is deep bluish green. The color of the *pallidum* is
bluish red in osmic acid preparations, and the organ-
ism appears thicker than in Giemsa preparations
otherwise fixed.

Old Giemsa preparations which have faded on standing may be restained by removal of the cedar oil mount and application of the regular technique. Overstained preparations may be differentiated by quickly passing through a 10 per cent *glycerinether-mischung* (Grübler) and rinsed in water or by first moistening the film with distilled water and then passing the slide swiftly through acetone and again through distilled water. Kraus, and later Hoffmann, found that 50 to 60 per cent tannin solution will clear the overstained background without decolorizing the spirochetes. In general a badly stained Giemsa preparation can seldom be redeemed. If a differentiated preparation fails to show the spirochetes the conclusion should not be drawn that they are not present. I have found such preparations to give satisfactory positive results, however, when restained by Fontana's method.

2. *Mordant Staining* (Figs. 18a, 21e, 23,i,j,k,l, 24g, 26c, 27d)

All methods which stain the bacterial flagella can be used to demonstrate *Treponema pallidum;* not only the body, but also the terminal flagella, if present, can be demonstrated. Needless to say, all precautions, chemical cleanliness, thinness of film, etc., prescribed for successful flagella staining, must be carefully observed. Patience and skill are important factors. Loeffler's, Van Ermengen's, and Cesares-

Gil's methods are all suitable. Miss Tilden has found that a suitable ink, such as that sold for use in fountain pens (Waterman) may be substituted for Loeffler's mordant with satisfactory results (Fig. 18a).

The following method (Figs. 21e; 23, i–l; 24g; 26c; 27d) has often given good results though not constantly: The film is fixed in methyl alcohol for 15 minutes, then, after being washed in water, is covered with a solution of mordant (5 per cent tannin in 1 per cent phenol) and held over a gentle flame for 1 minute, during which time it begins to steam. It is again washed in running water, covered with a strong aqueous solution of gentian violet to which 1 per cent phenol has been added, and steamed briefly over a flame, washed well in water, and air dried. Care must be taken not to make too thick a film.

3. Staining of Preparations Fixed in Buffered Formalin (Figs. 18b, 21f)

Treponema pallidum in film preparations stains well with basic fuchsin and gentian violet if previously fixed in buffered formalin[1] solution (Miss Tilden's method), which is made as follows:

> Formalin.................................... 10 c.c.
> Buffer phosphate solution........................ 90 c.c.
> (consisting of 88 c. c. $M/15$ Na_2HPO_4 + 12 c. c. $M/15$ KH_2PO_4)

[1] Throughout this chapter the commercial name, formalin, is used for the 40 per cent solution of the gas. Hence any reference to 10 per cent formalin means 1 part of the 40 per cent solution to 9 parts of diluent.

The scraping, exudate, or tissue emulsion is suspended in a small amount of the fixing solution and the mixture allowed to stand for at least 5 minutes. Thin films are made and allowed to dry in the air, or a drop of the fixative (from a capillary pipette) may be put on a slide and a drop of the exudate, or other material added, the mixture allowed to stand for 5 minutes or longer, protected from evaporation, and then spread into a *thin film* and dried in the air. The dry film surface is flooded with the staining solution, which is left for 30 to 60 seconds, then washed off in running water. Giemsa solution may also be used after buffered formalin fixation, any one of the procedures described (p. 292) being applicable.

The organisms in tissues remain well preserved for months in buffered formalin solution and may be teased out for darkfield examination or for staining by the method just outlined. They take a more intense stain when teased out of tissues.

Preparations made from solid (agar) cultures, which are difficult to stain by other procedures, have been stained successfully by this method.

STAINING OF SECTIONS

All methods based upon the precipitation of reduced silver on spirochetes in tissues are alike in giving inconstant results. In hundreds of instances in which we can demonstrate the *pallidum* in enor-

mous numbers under the darkfield microscope—e.g., in experimental syphilitic orchitis or chancre in the rabbit—the sections impregnated by various methods fail to show the organisms. Hence a negative finding by silver impregnation of tissues should never be taken as proof that the organisms were not present. The factors which determine the success or failure of these methods are still not understood, and further investigation is desirable. In the writer's experience, the demonstration of the *pallidum* in *post-mortem* materials by silver impregnation has been more frequent and consistent than in tissues excised during life.

The typical method is that first described by Bertarelli and Volpino and later improved by Levaditi.

1. *Levaditi's Method* (Figs. 18*f*; 21*g*; 22*b*)

Fix thin pieces of tissue (3 to 4 mm.) in 10 per cent formalin solution for 24 hours.

Absolute alcohol for 24 hours.

Distilled water until the tissue sinks.

Impregnate in a brown bottle with 1.5 to 3 per cent silver nitrate solution for 3 days in the incubator.

Wash for 15 to 30 minutes with distilled water.

Reduce for 24 hours in the dark at room temperature in a solution of

Pyrogallic acid	4 gm.
Formalin	5 c.c.
Distilled water	100 c.c.

Wash in distilled water, dehydrate (80 per cent alcohol, absolute alcohol, xylene), and embed in paraffin. Cut in thin sections, mount in balsam. The spirochetes are dark brown or black against a yellow background. Toluidin blue or Giemsa solution may be used as a contrast stain.

2. *Levaditi-Manouélian Method*

Fix thin pieces of tissue 24 hours in 10 per cent formalin and harden by putting in 90 per cent alcohol for 24 hours. Wash with distilled water until the tissue sinks to the bottom of the container.

Impregnate in a dark bottle for 2 to 3 hours at room temperature and for 3 to 5 hours in the incubator in the following solution:

<div style="margin-left:2em">

1 per cent silver nitrate solution................. 90 c.c.
Pure pyridine.................................... 10 c.c.

</div>

Wash in distilled water. Place in the following solution, freshly prepared, at room temperature for 4 hours:

<div style="margin-left:2em">

4 per cent aqueous pyrogallic acid solution......... 90 c.c.
Pure acetone.................................... 10 c.c.
Pyridine.. 17 c.c.

</div>

Dehydrate in absolute alcohol, clear in xylene, and embed in paraffin.

3. *Noguchi's Method for Nerve Tissue* (Fig. 19)

Fix in 10 per cent formalin, using pieces 5 to 7 mm. thick. The longer the fixation the better the differentiation between spirochetes and nerve fibers.

Leave for 5 days at room temperature in the following solution:

Formalin... 10 c.c.
Pyridine... 10 c.c.
Acetone.. 25 c.c.
Absolute alcohol... 25 c.c.
Distilled water.. 30 c.c.

Wash in distilled water for 24 hours, changing frequently, then place in 95 per cent alcohol for 3 days. Wash again in distilled water, with frequent changes, for 24 hours.

Impregnate in a dark bottle with 1.5 per cent silver nitrate solution for 3 days at 37°c. or for 5 days at room temperature. Wash thoroughly in distilled water.

Reduce for 24 to 48 hours at room temperature in a dark bottle with 4 per cent pyrogallic acid containing 5 per cent formalin. Wash thoroughly in distilled water.

Dehydrate with 80 per cent alcohol for 24 hours, 95 per cent alcohol for 3 days (with frequent changes), and absolute alcohol for 2 days. Clear in xylene, and embed in paraffin.

Sections should be made from different depths of the block in order to obtain a zone of optimum impregnation.

4. *Jahnel's Method*

Small blocks fixed in 10 per cent formalin (preferably for at least 14 days) are placed in pure pyridine for 1 to 3 days, thoroughly washed in water, left in 5 to 10 per cent formalin for a few days or until the pyridine has been removed, then washed again in water. Treatment with freshly prepared 1 per cent uranium nitrate in distilled water for ½ to 1 hour at 37°c. follows, the purpose being to minimize the

staining of nerve fibers in the subsequent impregnation with silver. Thorough penetration of the uranium nitrate from all sides is insured by putting a piece of glass wool in the bottom of the bottle. The blocks are now washed in distilled water for 24 hours, then transferred to 95 per cent alcohol for 3 to 8 days, and again washed in distilled water, this time only until they sink to the bottom of the container.

The silver impregnation is carried out with a 1.5 per cent solution of silver nitrate in a dark bottle in the incubator for 5 to 8 days. It is important always to use plenty of silver solution and not to put too many blocks into the bottle. Pure crystals (Merck) should be used in making the solution.

Decant the silver solution, rinse in distilled water and reduce for 1 to 2 days in the same dark bottle at room temperature in 4 per cent pyrogallic acid solution containing 5 per cent formalin. The solution should be freshly made and the pyrogallic acid pure. The tissues are not exposed to the light from the time they are placed in the silver solution until after reduction is completed.

Wash in distilled water, dehydrate by passage through 80 per cent, 95 per cent, and absolute alcohols, clear in xylene, and embed in paraffin.

5. Manouélian's Method

Fix pieces of tissue 1 mm. thick in 10 per cent formalin for 1 hour, wash three times in 90 per cent

alcohol during 10 minutes, and immerse in distilled water until the fragments fall to the bottom.

Impregnate with 1 per cent silver nitrate solution for 1 hour at 55°c. The solution should be at room temperature when the pieces of tissue are introduced, then transferred to the thermostat. Wash in distilled water for 5 minutes.

Immerse in the following mixture for 1 hour:

Pyrogallic acid, 2 per cent, in distilled water................... 90 c.c.
Formalin.. 10 c.c.
    Martin and Pettit have modified Manouélian's reduction bath by using the following mixture:

Pyrogallic acid..............................	1	gm.
Tannin.....................................	0.6	gm.
Sodium acetate..............................	2.0	gm.
Distilled water..............................	70.	c.c.

Dehydrate and embed in paraffin as usual.

6. *Warthin's Modification of Levaditi's Method*

This method is superseded by Warthin and Starry's silver-agar cover glass method (q. v.).

Tissues must be as fresh as possible, may even be fixed while still warm, and the blocks should not be more than 5 mm. thick.

Fix for 3 to 10 days in the following:

Sodium chloride...........................	0.9	gm.
Sodium bicarbonate........................	0.01	gm.
Potassium chloride........................	0.02	gm.
Calcium chloride..........................	0.02	gm.
Formalin.................................	10.	c.c.
Distilled water............................	100.	c.c.

The formalin must be colorless and free from tannic acid. A perfectly neutral formalin solution (10 per cent) may also be used for fixation. The longer the fixation the better the silver impregnation.

Wash in frequently changed distilled water for 24 hours, and after-harden in 90 per cent alcohol for 3 days. Wash again in frequently changed distilled water for 24 hours.

Impregnate in 2 per cent silver nitrate solution in the dark for 3 days in the incubator.

Wash thoroughly in distilled water in the dark.

Reduce for 48 hours in the dark at room temperature in a solution containing 4 per cent pyrogallic acid and 5 per cent formalin in distilled water.

Wash thoroughly in distilled water.

Dehydrate, beginning with 80 per cent alcohol for 24 hours, and passing to 95 per cent and then absolute. Embed in paraffin.

7. *Haythorn's Method*

Pieces of tissue from 2 to 3 mm. in thickness are fixed in 10 per cent formalin solution for 8 hours or over night, transferred to warm distilled water (50 to 60°c.) for 10 minutes, laid on clean filter paper just long enough to take off the excess of water, then placed for ½ hour in chemically pure acetone. The pieces are incubated for 8 hours or over night in Solution 1:

10 per cent silver nitrate solution	5 c.c.
Acetone (c. p.)	10 c.c.
Distilled water	5 c.c.

mixed just before use and filtered. On removal from Solution 1 the tissues are touched to clean filter paper and transferred to a clean bottle containing freshly prepared and filtered Solution 11:

Pyrogallic acid	1 gm.
Acetone (c. p.)	25 c.c.

They are allowed to remain in Solution 11 from 6 to 8 hours and then passed through the following solutions in the order named: Absolute alcohol, 2 hours or more; cedar wood oil, 1 hour; xylene, ½ hour; paraffin, 2 hours. They are then blocked. The

sections are cut as thin as possible and the first ten or fifteen discarded, because the tissue is thoroughly penetrated, and there is sometimes a precipitate on the surface. All traces of paraffin are removed with xylene, and the sections are mounted in balsam. The spirochetes appear black.

8. Gyenes[1] and Sternberg's Method

Blocks should be well fixed in 10 per cent formalin. Sections 5–8–10 micron thick are cut either from blocks embedded in celloidin or from frozen blocks. Frozen sections are washed 2 to 3 minutes in distilled water; celloidin sections are passed through alcohol and then through distilled water.

Place sections in 1 per cent silver nitrate solution in the incubator (37°c.) for 30 to 35 minutes. Transfer to a 2 per cent solution of silver nitrate (10 parts) to which are then added the following:

5 per cent gelatin solution.................................... 10 c.c.
50 per cent gum arabic solution.............................. 10 c.c.

Mix well and add 5 c. c. of a 5 per cent solution of hydroquinone. When sections become dark brown remove them, before the reduced silver is precipitated, and fix in 10 per cent sodium thiosulphate (1 to 2 minutes). Wash in distilled water, dehydrate (95 per cent alcohol, absolute alcohol, chloroform-alcohol

[1] In several textbooks the spelling Eyenes is found, but reference to the original article shows Gyenes to be the correct form of the name.

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equal parts, terpineol-chloroform-alcohol equal parts, Canada balsam).

9. *Warthin and Starry's Coverglass Method (forerunner of their silver-agar coverglass method)*

Fix tissues in 10 per cent formalin.

Wash thoroughly in distilled water. Embed in paraffin (alcohol, xylene, paraffin), cut sections, and mount on coverglasses with albumin fixative.

Remove paraffin from section (xylene, alcohol, water) and place coverglass in a saturated solution of ferric alum or a 4 per cent solution of ferrous ammonium sulphate in the incubator for 1 to 2 hours.

Wash in distilled water.

Rinse coverglass with section in a 2 per cent silver nitrate solution. Cover section with another perfectly clean coverglass which has also been rinsed in the silver solution, so that the coverglasses are held together by capillary attraction. Then place them carefully on the bottom of a wide-mouthed dark bottle covered with dark paper and cover them with the silver nitrate solution. Cork tightly and put into the incubator for 3 to 24 hours.

After impregnation pour off the silver nitrate solution and rinse in distilled water, without removing coverglasses from bottle, by pouring the water into the bottle, shaking gently, and then pouring off.

Pour the reducing fluid (pyrogallic acid, 4 gm.; formalin, 5 c. c.; distilled water 100 c. c.) into the bottle. See that the fluid passes between coverglasses by pressing upon them with a glass rod, or by shaking. Reduction is almost instantaneous; it should occur evenly over the section, or brown lines will result. After 2 to 3 minutes remove coverglasses, separate, wipe off with a cloth any precipitate on the albumin fixative about the section, taking care not to touch the latter.

Wash in distilled water, dehydrate in absolute alcohol, clear in xylene, mount in balsam. The reduced section should have a faint, dull brownish-yellow color. If the color is bright yellow the organisms will be poorly stained. The spirochetes should have a deep reddish brown color contrasting sufficiently well with the background, if the procedure has been successful.

10. *Warthin and Starry's Silver-agar Coverglass Method*

This method is recommended by Warthin and Starry as superior to their earlier methods.

Fix tissue in 10 per cent neutral formalin. Embed in paraffin (absolute alcohol, xylene, paraffin), cut, and mount sections on coverglasses with albumin fixative.

Remove paraffin (xylene, alcohol, water).

Rinse coverglass with section in 2 per cent silver nitrate; cover wet section with another perfectly clean coverglass, so that they are held together by capillary attraction, place them carefully in a bottle of 2 per cent silver nitrate and put in the incubator for 30 to 60 minutes, then remove the silver nitrate and separate the coverglasses. They must not separate while in the solution.

Place coverglass with section in the following reducing mixture:

2 per cent silver nitrate solution.............................	3 c.c.
Warm glycerol	5 c.c.
Warm 10 per cent aqueous gelatine.........................	5 c.c.
Warm 1½ per cent agar suspension[1]........................	5 c.c.
5 per cent aqueous hydroquinone solution......................	2 c.c.

Reduce until section is a slight reddish brown (several seconds), remove, and rinse in 5 per cent sodium thiosulphate solution.

Rinse in distilled water. Clean off with a cloth any precipitate on the coverglass.

Absolute alcohol, xylene, balsam.

The mounted section should have a light reddish brown color; if too deep brown or black the spirochetes will not be sufficiently contrasted. They should

[1] See footnote, p. 289.

appear dark reddish brown to jet black against a very light brown background.

In some cases of poor fixation of the tissue with poor staining of the spirochetes, Warthin and Starry state that if the sections, before being put into the silver solution, were treated for several minutes with a 1 per cent solution of uranium or copper nitrate or a 0.5 per cent solution of ferric alum, the tissue-reaction was changed in some way so that good staining resulted. The solutions did not act uniformly in all cases. After their use the section must be thoroughly washed in distilled water before it is put into the silver nitrate solution.

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DEFINITIONS OF TERMS USED IN IMMUNOLOGY

AGGLUTINATION. Clumping of bacteria or blood-corpuscles by specific agglutinins. For corpuscles the term hemagglutination is often used.

AGGLUTININS. A single or repeated injection of bacteria or foreign blood corpuscles into an animal is followed by the development of a new property in the serum of that animal. This serum, when deprived of its own complement either by inactivation or by dilution, is capable of clumping in the test tube the bacteria or blood corpuscles employed for immunization. This phenomenon is called agglutination and is ascribed to the reaction product designated agglutinin. Its nature is not known except that it is found in the protein fraction of serum and resists the temperature of 56°c. Agglutinins may be found in some normal sera in varying quantity. Antibodies, being agglutinins, are specific, and can be absorbed by bacteria or blood-corpuscles.

ALEXIN. Term first introduced by Hans Buchner, adopted by Bordet; is now synonymous with complement of Ehrlich and cytase of Metchnikoff. See Complement. Buchner's idea of alexin is not identical with that of Bordet, and the term was used to designate bacteriolysins and hemolysins but not the complement of Ehrlich or the alexin of Bordet. The term alexin to-day is used in Bordet's sense, not in Buchner's.

AMBOCEPTOR. Term introduced by Ehrlich; is synonymous with *fixateur* of Metchnikoff, *substance sensibilisatrice* of Bordet, *preparator* of Max Gruber, and *copula* of P. Th. Müller. Amboceptor is one of the two active principles necessary to cause hemolysis, bacteriolysis, or any other cytolysis caused by serum, the other active principle being complement. Amboceptor retains its activity after the serum is heated to from 55° to 56°c. for 30 minutes, while complement is destroyed at that temperature. Amboceptor, as well as complement, is present in the coagulable protein fraction of serum. Amboceptor may be present in any

normal serum, and can be produced in the serum of an animal by injecting repeatedly the cells for which it has no amboceptor. The amboceptor normally present is called natural amboceptor and that which is produced by means of repeated injections of foreign cells is called immune amboceptor. The amboceptor capable of causing hemolysis (in presence of complement, of course) is called hemolytic amboceptor, while that which is capable of dissolving bacteria is called bacteriolytic amboceptor. A few writers use the simple terms of hemolysin or bacteriolysin instead of hemolytic or bacteriolytic amboceptor. Amboceptors are capable of producing anti-amboceptors when injected into a susceptible animal.

ANTIBODIES. A general term applied to a group of reaction products arising from single or repeated administrations of antigens to a suitable animal. Immune body is a synonym of antibody. Among antibodies we may enumerate hemolytic amboceptors, bacteriolytic amboceptors, other cytolytic amboceptors, precipitins, agglutinins, antitoxins, antivenins, antiricin, antiabrin, etc. Antibodies possess specific affinity for the antigens which are used for their production. Certain antibodies such as agglutinins, amboceptors, antitoxins, or antihemolysins may be normally present in certain sera in small amount. A group of antibodies is capable of producing antibodies when injected into another animal, thus forming anti-antibodies.

ANTICOMPLEMENTARY ACTION. Substances possessing the power of reducing or removing totally the action of complements are said to be anticomplementary. Most acids, alkalies, and certain salts have anticomplementary action. In some sera there are often certain principles possessing anticomplementary properties. Human serum gradually acquires this property on standing. Repeated injections of fresh serum into an animal of another species is followed by the appearance of anticomplements (Ehrlich and Morgenroth); Gay considers this phenomenon an example of complement-fixation by specific precipitate.

ANTIGENS. A general term applied to a group of substances capable of producing specific antibodies, when administered once or repeatedly, usually by injection, to a suitable animal. For example, bacteria, blood-corpuscles, and certain somatic cells are antigens because they produce specific antibodies called amboceptors and agglutinins. Blood-serum, milk and bacterial extracts are also antigens, because they produce antibodies called precipitins, each being specific for the substance employed for its production. On the other hand, most inorganic or organic substances with definite chemical structure are not antigens, because their introduction is not followed by the formation of antagonistic substances (antibodies) in the body. Repeated administrations of various alkaloids render the organism gradually more resistant to their effect, but do not produce antibodies, hence these alkaloids are not antigens. Diphtheria toxin, tetanus toxin, ricin, abrin, snake venoms, are antigens, and their injections are followed by specific antitoxins, as is well known.

BACTERIOLYSINS. Active principles in blood-serum capable of dissolving bacteria and, consisting of specific bacteriolytic amboceptors and complement. Analogous to hemolysins and cytolysins in general.

BACTERIOLYSIS. Dissolution of bacteria by immune or normal sera. It is caused by specific bacteriolytic amboceptors and complement. Analogous in mechanism to hemolysis.

BACTERIOTROPINS. Term introduced by Neufeld to denote active principles of certain immune sera inducing phagocytosis. Their action is on the bacteria but not on the phagocytes. They are thermostable.

BACTERIOPHAGE. Term introduced by d'Herelle to designate bacteriolytic substances derived from the dissolved bodies of certain bacteria after the latter have come into contact with immune substances *in vitro*. The bacteriophage is transmitted by the bacteria which resist its dissolving action. It is filterable.

COMPLEMENT. Term introduced by Ehrlich; is synonymous with Metchnikoff's cytase and Bordet's alexin. By the term complement one understands one of the two active principles concerned in hemolysis, bacteriolysis, and other instances of serum cytolysis. The other principle, called amboceptor, is incapable of causing dissolution of cells without complement, hence the latter term. Complement is normally present in all sera freshly drawn from the body, but disappears gradually on standing or is completely destroyed at from $55°$ to $56°$c. in about thirty minutes. Complement of one species is not identical in its action with that of other species.

COMPLEMENT DEFLECTION. Synonymous with complement deviation.

COMPLEMENT DEVIATION. Synonymous with deflection; originated from a German term *Ablenkung*, introduced by Neisser. Complement deviation is identical with *Komplementbindung* of the Germans; Bordet uses the term deviation or fixation of alexin. By the deviation of complement one understands that complement is rendered inactive by the antigen-antibody combination and is made unavailable for a second set of antigen-antibody combination to complete a reaction in which complement is essential. This second set may be a hemolytic or a bacteriolytic system. See illustrations on pp. 22 and 23.

COMPLEMENT FIXATION. Synonymous with complement deviation.

COMPLEMENTOIDS. Modified complements in which the zymotoxic group is destroyed but not the group capable of uniting with amboceptors. Complementoids are formed at $56°$c.

COMPLEMENTOPHILIC GROUP. Atom-complex of amboceptor to which complement attaches. This complex remains inactive until the cytophilic group (another atom-complex) of the amboceptor joins with the receptor of the cell.

COPULA. Synonymous with amboceptor.

CYTASE. Introduced by Metchnikoff; is synonymous with

alexin of Bordet and complement of Ehrlich. See
Complement.

CYTOLYSINS. Active substances in blood-serum consisting of
specific cytolytic amboceptors and complement.

CYTOLYSIS. Dissolution of cells by specific amboceptors and
complement. In the case of blood-corpuscles the term
hemolysis is used and in that of bacteria the term bacterio-
lysis is used.

CYTOPHILIC GROUP. Atom-complex of amboceptor with which
the receptor of a cell unites. Thus an amboceptor possesses
two atom-complexes, one for the complement and the
other for the receptor of the cell.

ENDOTOXIN. Toxic constituents of bacterial cells.
EXOTOXIN. Soluble toxic products of bacteria.

FIXATEUR. Metchnikoff's term for *amboceptor* of Ehrlich and
substance sensibilisatrice of Bordet. See Amboceptor.

HEMOLYSINS. Any substance capable of causing hemolysis
may be called an hemolysin, but its use is restricted to
the biological products of unknown chemical constitution,
especially the blood-serum; in particular, the amboceptor
of the serum is often called hemolysin.

HEMOLYSIS. Dissolution of blood-corpuscles by various agents,
setting the hemoglobin free in the medium in which the
corpuscles are suspended. Distilled water, freezing and
thawing, temperature of about $55°$C. for thirty minutes,
etc., are physical agents which cause hemolysis. Acids,
alkalies, and certain salts can cause hemolysis in proper
concentrations. Of these chemicals may be mentioned
most organic acids, mineral acids, all alkalies, bile salts,
bichloride of mercury, soaps. Of biological origin may be
mentioned certain glucosides such as saponin, solanin, etc.,
certain bacterial cultures such as those of staphylococcus,
vibrios, megatherium, tetanus bacillus, etc.; certain
animal venoms such as those of snakes, bees, spiders, etc.

The hemolytic process caused by these different agents is different according to the nature of the hemolytic agents, but all attack the corpuscles more or less directly. Hemolysis by serum is, however, somewhat different from that caused by the various factors just mentioned. For example, hemolysis by fresh alien serum is caused by two distinct groups of substances, both contained in blood-serum. One is called complement and the other amboceptor. The one is inactive without the other. Serum hemolysis forms the basis of many interesting phenomena, the serum diagnosis of syphilis being one of these.

HEMOLYTIC AMBOCEPTORS. See Amboceptors.

HAPTINS. Term introduced by Ehrlich; synonymous with antibodies, used in somewhat broader sense.

HAPTOPHORE GROUP. The atom-complex of complement which is capable of uniting with the complementophilic group of amboceptor; complement is thus united with the cell through the intermediation of amboceptor.

IMMUNE BODIES. Synonymous with antibodies.

INACTIVATION. Fresh serum containing both amboceptor and complement becomes inactive when heated to from 55° to 56°c. for about 30 minutes because of the destruction of complement. This process is called inactivation, and the heated serum is called inactivated serum. Amboceptor is not affected materially by the process.

INTER-BODY. Ehrlich used the term *zwischenkörper* before he introduced the term *amboceptor*, and its English version is *inter-body* (Bolduan) or *intermediary body* (Flexner and Noguchi).

INTERMEDIARY BODY. Synonymous with inter-body.

ISO-AGGLUTININ. Blood-serum of an animal usually does not contain agglutinins for the blood-corpuscles of another animal of the same species, but in some instances agglutination may occur and is due to the substances called iso-agglutinins.

ISO-HEMOLYSIN. Blood-serum of one animal usually does not

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hemolyse the blood-corpuscles of another animal of the same species, but in some instances hemolysis may occur. This phenomenon is known as iso-hemolysis and is caused by the presence of iso-hemolysin. In man this is observed quite frequently in the serum of patients suffering from malignant tumors.

OPSONINS. Term introduced by Sir Almroth Wright to designate active substances, of normal as well as immune sera, which cause phagocytosis. Normal opsonins are rendered inactive at 56°C. and seem to depend upon the cooperation of complement. Immune opsonins are thermostable.

PLEOCYTOSIS. Term introduced by Nonne and identical with lymphocytosis in the cerebrospinal fluid in syphilitic and parasyphilitic diseases of the central nervous system.

PRECIPITATES. By the term precipitate in immunological terminology is meant the flocculation or clumping brought about by mixing specific antigen and antibody, such as serum precipitates, bacterial extract precipitates, etc.

PRECIPITATION. In immunological terminology one understands by precipitation a clumping phenomenon of protein or protein-like substances by specific precipitins.

PRECIPITIN. In the blood-serum of an animal which has received repeated injections of a solution of protein matter there is found a substance capable of precipitating that protein when mixed in a test tube. This precipitating principle is called precipitin, and its action is specific; that is, a precipitin for human serum precipitates the latter, but no other serum. Precipitins can be produced in animals for different proteins, such as egg albumin, serum, milk, bacterial proteins, etc. It is resistant to the temperature of 56°C. like most immunization products, and remains active for a very long time when desiccated.

PRECIPITINOGEN. A general term occasionally used for the substances capable of producing precipitins by means of immunization, that is, repeated injections into animals.

PREPARATOR. Synonymous with amboceptor.

PROTECTIN. A term introduced by the writer to designate a substance (or substances) developing in all blood-sera on standing *in vitro*, and characterized by its property of protecting blood-corpuscles against hemolytic serum. This protective substance (or substances) is taken up by the corpuscles through long contact, the property being increased in sera of certain animals after heating to 60°C. or a little higher. It is similar to the complementoid of Ehrlich and Morgenroth, differing only in its capability of being absorbed by nonsensitized cells and extracted by fat solvents such as ether, acetone, and alcohol.

REACTIVATION. The addition of complement to an inactivated serum restores its lytic activity, and the process is called reactivation.

RECEPTORS. Constituents of the cell uniting with amboceptors or any other antibodies or toxins. The presence or absence of receptors determines whether or not the cell is susceptible to a given amboceptor or toxin.

SENSIBILIZATION (French). Synonymous with sensitization and caused by allowing amboceptor to act on cells.

SENSITIZATION. When a cell is acted upon by specific amboceptor it becomes sensitive to the dissolving action of complement. This process of rendering a cell sensitive is called sensitization. In French it is sensibilisation.

SENSITIZER. Synonymous with *amboceptor* of Ehrlich and *substance sensibilisatrice* of Bordet.

SENSITIZING SUBSTANCE. Synonymous with *amboceptor*.

STIMULIN. Term introduced by Metchnikoff; is an active principle of serum inducing phagocytosis. Metchnikoff thought it due to the stimulation of phagocytes, but it is probably identical with the opsonins of Wright.

SUBSTANCE SENSIBILISATRICE. Term introduced by Bordet; is synonymous with Ehrlich's *amboceptor*, Metchnikoff's *fixateur*, Gruber's *Preparator*, or P. Th. Müller's *copula*. Bordet often uses the term "sensibilisatrice." See Amboceptor.

THERMOLABILE. Complement loses its activity at about 55° to 56°c. in about thirty minutes and hence is called thermolabile.

THERMOSTABLE. Amboceptor remains still active after the serum containing it is heated at 55° to 56°c. for thirty minutes and hence is said to be thermostable.

TOXINS. A general term for a group of substances, chiefly of bacterial elaboration, possessing a powerful toxicity and one or more of the following characteristics. Thermolability, incubation period for action, unknown chemical constitution, difficulty in separation from protein molecule, capability of producing antibodies. The best known examples are diphtheria toxin and tetanus toxin, both of which are true toxins and of extracellular origin (exotoxins). The toxic principles of cholera vibrio, meningococcus, gonococcus, typhoid bacillus, dysentery bacillus are chiefly contained in the cell-body and are called endotoxins. Tuberculin is an atypical, extracellular toxin. Toxalbumins of higher plants and toxic secretions of snakes, spiders, and bees resemble bacterial toxins in many respects.

TOXOIDS.

Ehrlich modified various toxins by chemicals in such a manner as to reduce or remove their toxic property without destroying their immunizing property. The modified toxins are called toxoids. They may arise spontaneously under certain circumstances and combine, like toxins, with antitoxins.

TOXOPHORE GROUP. Analogous with zymotoxic group of complement.

ZYMOTOXIC GROUP. Analogous with toxophore group of a toxin and represents the active dissolving atom-complex of a complement. The destruction of this group leads to the formation of a complementoid.

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